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Cofactor Treatment in Oxidative Phosphorylation Disorders

by

Barbara J Marriage

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Cofactor Treatment in Oxidative Phosphorylation Disorders* submitted by Barbara Marriage in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences - Medicine

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ABSTRACT

Marked progress has been made over the past 15 years in defining the specific biochemical defects and underlying molecular mechanisms of oxidative phoshorylation defects, but limited information is currently available on the development and evaluation of effective treatment approaches. Metabolic therapies that have been reported to produce a positive effect include coenzyme Q_{10} (ubiquinone), other antioxidants (ascorbic acid, vitamin E), riboflavin, thiamine, niacin, vitamin K (phylloquinone and menadione), and carnitine. The goal of therapy is to increase mitochondrial ATP production, and to slow or arrest the progression of clinical symptoms. We have developed a method that utilizes circulating lymphocytes to examine the ability of the mitochondria to synthesize ATP when provided with selected substrates. In the present study, we have been able to demonstrate for the first time that an increase in ATP synthetic capacity in lymphocytes results from cofactor administration. To determine the effect of the individual components of the cofactor treatment on ATP synthesis we examined *in vitro* cofactor supplementation in control lymphocytes. A dose-dependent increase in ATP synthesis with CoQ_{10} incubation was demonstrated. Our data suggest that CoQ_{10} may have a beneficial effect in the treatment of OXPHOS disorders. The rapid and easy determination of ATP synthesis in lymphocytes from patients with mitochondrial disorders will allow designing of the most appropriate therapy on an individual basis.

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TABLE OF CONTENTS

Chapter 1

Introduction 1
Oxidative Phosphorylation (OXPHOS) 2
Genetics of OXPHOS Defects 5
Nuclear OXPHOS Gene Mutations 12
Mitochondrial DNA OXPHOS Mutations 16
Nuclear-Mitochondrial Communication Disorders 21
Indirect Effects on OXPHOS 23
Diagnostic Evaluation in OXPHOS Disorders 24
Metabolic Investigations 24
Molecular Testing 25
Histochemical Evaluation 26
Spectrophotometric Studies 26
Polarographic Studies 28
ATP Synthesis 29
Summary 30
References 31

Chapter 2

Cofactor Treatment in OXPHOS Disorders 43
Introduction 43
Coenzyme Q_{10} (Ubiquionone) 43
Antioxidants 51
Vitamin C 51
Vitamin E 52
Riboflavin 53
Thiamine 56
Niacin 57
Vitamin K (Phylloquinone and Menadione) 59
Carnitine 61
Combined Therapy Treatments 65
Summary 66
References 73

Chapter 3

Research Plan 82
Rationale 82
Goal 87
Objectives 88
References 91

Chapter 4

Use of Lymphocytes to Screen for OXPHOS Disorders 94
Introduction 94
Materials and Methods 97
Results and Discussion 100
References 116

Chapter 5

The Effect of Cofactor Treatment on ATP Synthetic Capacity in Patients with OXPHOS Disorders 122
Introduction 122
Materials and Methods 124
Results 128
Discussion 142
References 148

Chapter 6

The Effect of Cofactor Incubation on ATP Synthetic Capacity in Control Lymphocytes 153
Introduction 153
Materials and Methods 154
Results 156
Discussion 166
References 173

Chapter 7

Summary and Conclusions 177
References 183

APPENDICES

Appendix I Informed Consent and IRB Approval.....184

Appendix II Clinical History of Patients.....188

Curriculum Vitae.....195

List of Tables

Table 2.1 Coenzyme Q_{10} Treatment in OXPHOS Disorders 69

Table 2.2 Vitamin and Cofactor Treatment in OXPHOS Disorders 71

Table 4.1 Patient Clinical and Diagnostic Data 98

Table 4.2 ATP Synthesis in Control Subjects and Patients..... 102

Table 5.1 Clinical and Diagnostic Data of Patients 125

Table 5.2 Plasma Coenzyme Q_{10} Levels 130

Table 5.3 ATP Synthesis in Control Subjects and Patients..... 133

Table 5.4 Effect of Cofactor Treatment on ATP Synthesis in LHON Patients..... 137

Table 5.5 Effect of Cofactor Treatment on ATP Synthesis in CPEO Patients..... 138

Table 5.6 Effect of Cofactor Treatment on ATP Synthesis in the MELAS Patient..... 139

Table 5.7 Effect of Cofactor Treatment on ATP Synthesis in the NARP Patient..... 140

Table 5.8 Effect of Cofactor Treatment on ATP Synthesis in the COX Deficiency Patient..... 141

Table 6.1 Comparison of Oral Supplementation and Incubation Dosages..... 155

List of Figures

Figure 1.1 Mitochondrial Respiratory Chain 6

Figure 1.2 Human Mitochondrial Genome Map 7

Figure 3.1 Measurement of ATP Synthesis in the Respiratory Chain 90

Figure 5.1 Plasma Coenzyme Q_{10} Levels 130

Figure 5.2 ATP Synthesis in Control Subjects and Patients..... 133

Figure 5.3 Effect of Cofactor Treatment on ATP Synthesis in Patients 134

Figure 5.4 ATP Synthesis in LHON Patients..... 137

Figure 5.5 ATP Synthesis in CPEO Patients..... 138

Figure 5.6 ATP Synthesis in the MELAS Patient 139

Figure 5.7 ATP Synthesis in the NARP Patient..... 140

Figure 5.8 ATP Synthesis in the COX Deficiency Patient 141

Figure 6.1 Effect of CoQ_{10} Incubation on ATP Synthetic Capacity..... 160

Figure 6.2 Time Course of CoQ_{10} and Carnitine on ATP Synthesis..... 161

Figure 6.3 Effect of Blocking ATP Synthesis with Oligomycin..... 161

Figure 6.4 Effect of Carnitine Incubation on ATP Synthetic Capacity .. 162

Figure 6.5 Effect of Vitamin B Incubation on ATP Synthetic Capacity . 165

Figure 6.6 Effect of Vitamin K Incubation on ATP Synthetic Capacity . 165

List of Abbreviations

ADP adenosine diphosphate

ANT adenine nucleotide translocase

ATP adenosine triphosphate

CoQ_{10} coenzyme Q_{10}

COX cytochrome *c* oxidase

CPEO chronic progressive external ophthamoplegia

CSF cerebrospinal fluid

DNA deoxyribonucleic acid

DPP deafness dystonia polypeptide

FAD flavin adenine nucleotide

FMN flavin mononucleotide

EBV Epstein Barr virus

ETF electron transfer flavoprotein

K_1 phylloquinone

K_3 menadione

KSS Kearns-Sayre syndrome

LHON Leber's hereditary optic neuropathy

LS Leigh syndrome

MELAS mitochondrial encephalopathy, lactic acidosis, stroke-like episodes

MERFF myoclonic epilepsy, lactic acidosis, ragged-red fibers

MNIGIE mitochondrial neurogastrointestinal disorder and encephalopathy

MRS magnetic resonance spectroscopy

mtDNA mitochondrial DNA

NADH nicotinamide adenine dinucleotide

NARP neurogenic ataxia and retinitis pigmentosa

NMR nuclear magnetic resonance spectroscopy

OXPHOS oxidative phosphorylation

PCR polymerase chain reaction

PCr phosphocreatine

P_i inorganic phosphate

POLG polymerase gamma

qid four times per day

RRF ragged-red fibers

rRNA ribosomal ribonucleic acid

tid three times per day

Tim translocase of the inner membrane

TMPD tetramethylphenylene diamine

CHAPTER 1

INTRODUCTION

Mitochondrial disorders are degenerative diseases characterized by a decline in the ability to supply cellular energy requirements. Oxidative phosphorylation (OXPHOS) is responsible for producing most of the adenosine 5'-triphosphate (ATP) that is required for cells. Defects in OXPHOS account for a large array of mitochondrial disorders, with onset occurring at any time. Mitochondrial diseases include encephalopathies, myopathies, neuropathies and cardiomyopathies, and are characterized by clinical, biochemical and genetic heterogeneity (Munnich and Rustin 2001). The clinical features vary extensively, depending on the etiology and severity of the mitochondrial dysfunction, age of onset, and tissues primarily involved in the disease (Peterson 1995). Disorders of oxidative phosphorylation may be as common as 1 in 5,000 to 1 in 10,000 births (Applegarth et al. 2000; van den Heuvel and Smeitink 2001). Marked progress has been made in the past 15 years in defining the specific biochemical defects and underlying molecular mechanisms of these defects, but limited information is currently available on the development and evaluation of effective treatment approaches. The goal of the work presented in this thesis is to develop objective measurements of the effect of cofactor treatment in mitochondrial disorders and to test the hypothesis that there will be an increase in cellular energy production after cofactor treatment.

OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation (OXPHOS) is carried out by five mitochondrial enzyme complexes, which are responsible for producing the bulk of cellular energy requirements. The assembly and maintenance of the nearly 100 polypeptides that make up the complexes are under the control of both nuclear and mitochondrial (mt) DNA genes (Shoffner 2001). These enzymes are situated in the inner mitochondrial membrane and are designated as complex I (NADH:ubiquinone oxidoreductase), complex II (succinate: ubiquinone oxidoreductase), complex III (ubiquinol: ferrocytochrome *c* oxidoreductase), complex IV (cytochrome *c* oxidase), and complex V (ATP synthase).

Complex I catalyzes the transfer of electrons from nicotinamide adenine dinucleotide (NADH) to ubiquinone through a series of redox groups, including flavin mononucleotide (FMN) and six iron sulphur groups (Shoffner 2001). As the electrons move through complex I, two protons are transferred to FMN to form $FMNH_2$; these protons are consumed in the reduction of ubiquinone to ubiquinol (Horton et al. 1996). The energy released is used to pump protons across the mitochondrial inner membrane. Complex I is composed of 42 polypeptides, seven of which are encoded by the mitochondrial DNA (mtDNA) (Shoffner 2001).

Complex II accepts electrons from the dehydrogenation of succinate to fumarate in the citric acid cycle and, in a way similar to complex I, catalyzes the reduction of ubiquinone to ubiquinol. Complex II

is situated on the matrix side of the mitochondrial membrane; because minimal free energy is released, it does not contribute to the proton gradient across the inner mitochondrial membrane. Complex II consists of four subunits — two of which contain succinate dehydrogenase, the flavin adenine nucleotide (FAD), and iron sulfur clusters; and two of which contain membrane-anchoring polypeptides (Shoffner 2001). Complex II is the only OXPHOS enzyme complex in which the subunits are entirely encoded by nuclear DNA.

Complex III catalyzes electron transfer between two electron carriers, ubiquinol and cytochrome *c*. The sequence of electron transfers produces a translocation of protons across the inner mitochondrial membrane. Complex III contains 11 polypeptides, including core proteins, iron sulfur proteins, cytochrome *b*, and cytochrome c_1. Cytochrome *b* is the only polypeptide encoded by mtDNA (Shoffner 2001).

Complex IV or cytochrome *c* oxidase (COX) is the last component involved in electron transport. This complex accepts electrons from reduced cytochrome *c* and donates them to oxygen, which is then reduced to water. Heme and copper cofactors are involved in the reduction of oxygen. Complex IV also translocates protons across the inner mitochondrial membrane and contains 13 polypeptides, three of which are encoded by mtDNA (Shoffner 2001). The mechanisms for both electron transfer and proton translocation are unclear.

Complex V utilizes the proton gradient generated by complexes I, III, and IV as a source of energy to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i). Complex V is composed of two segments: the F_1 component which catalyzes the hydrolysis of ATP, and the F_0 component which is a proton channel that spans the membrane and translocates protons into the mitochondrial matrix (Horton et al. 1996). Complex V is composed of 16 subunits, of which two are encoded by mtDNA (Shoffner 2001). Although ATP is synthesized in the matrix of the mitochondria, most is consumed in the cytosol. The mitochondrial ATP is exchanged for cytosolic ADP by a transporter called adenine nucleotide translocase (ANT) (Wallace et al. 2001).

OXPHOS is regulated by a variety of factors including cellular energy demand, substrate availability, oxygen supply, ion gradients and membrane transporters (Shoffner 2001). During normal metabolic processes, the rate-limiting step is usually the availability of ADP. In the presence of an oxidizable substrate with limiting ADP, oxygen uptake is slow; this is referred to as resting respiration. Electron transport and oxygen consumption increase when ADP is available accompanied by ATP formation; this state is referred to as active respiration. Because ADP is a rate-limiting substrate and the membrane transporter ANT exchanges mitochondrial ATP for cytosolic ADP, ANT may play a key role in the regulation of oxidative phosphorylation (Davis and Davis-van Thienen 1978; Kholodenko et al. 1987). The precise mechanisms underlying the

regulation of OXPHOS remain controversial. The relationship among respiratory complexes, electron flow, proton translocation and ATP production is illustrated (Figure 1.1).

GENETICS OF OXIDATIVE PHOSPHORYLATION DEFECTS

The genetics governing OXPHOS are complex because the genes encoding the polypeptides are found in both the nuclear and mitochondrial genome. In 1963, the presence of intramitochondrial fibers with DNA characteristics was observed for the first time; this marked the discovery of mtDNA (Nass and Nass 1963). Since that time, we have come to realize that multiple human diseases are a result of mtDNA mutations. The human mtDNA is a 16,569 base pair, double-stranded, circular molecule that encodes 13 OXPHOS subunits plus 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA) necessary for their expression (Lestienne and Bataille 1994) (Figure 1.2). The remaining polypeptides and proteins required for transcription, translation and replication of the mtDNA are all encoded by the nuclear genome. The genetics of OXPHOS are further complicated by the unique features of mtDNA inheritance, replicative segregation of the mtDNA molecule and the high mtDNA mutation rate (Shoffner 2001).

All mitochondrial DNA molecules in an individual are derived from the unfertilized ovum that gives rise to the zygote, which results in maternal inheritance. The egg contains several hundred thousand mitochondria and each mitochondrion contains two to ten DNA molecules

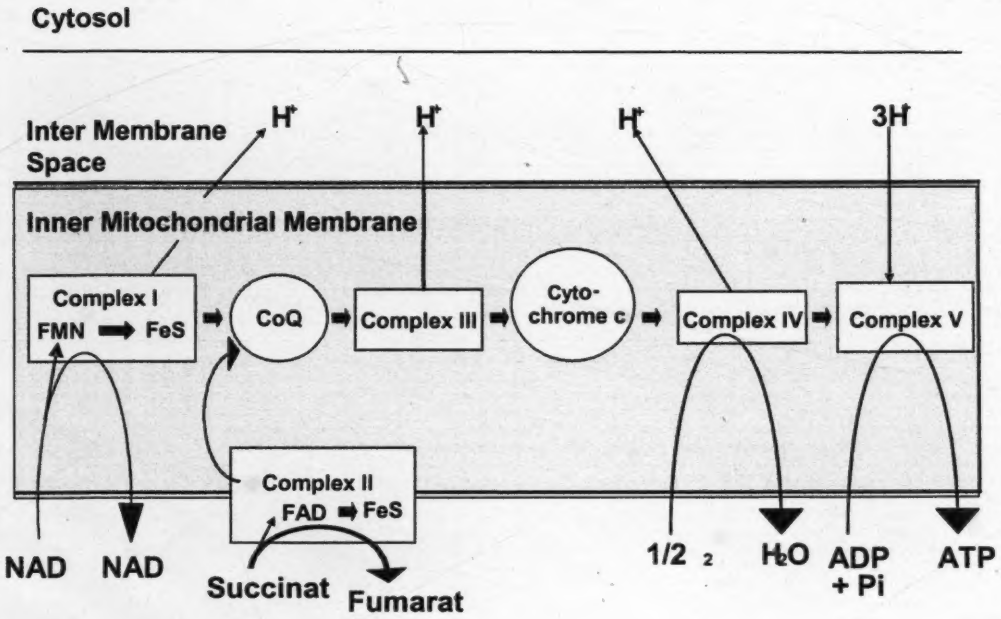

Figure 1.1 Mitochondrial Respiratory Chain
Protons (H^+) are pumped from the mitochondrial matrix to the intermembrane space through complexes I, III, and IV. Complex V utilizes the proton gradient as a source of energy to produce ATP.

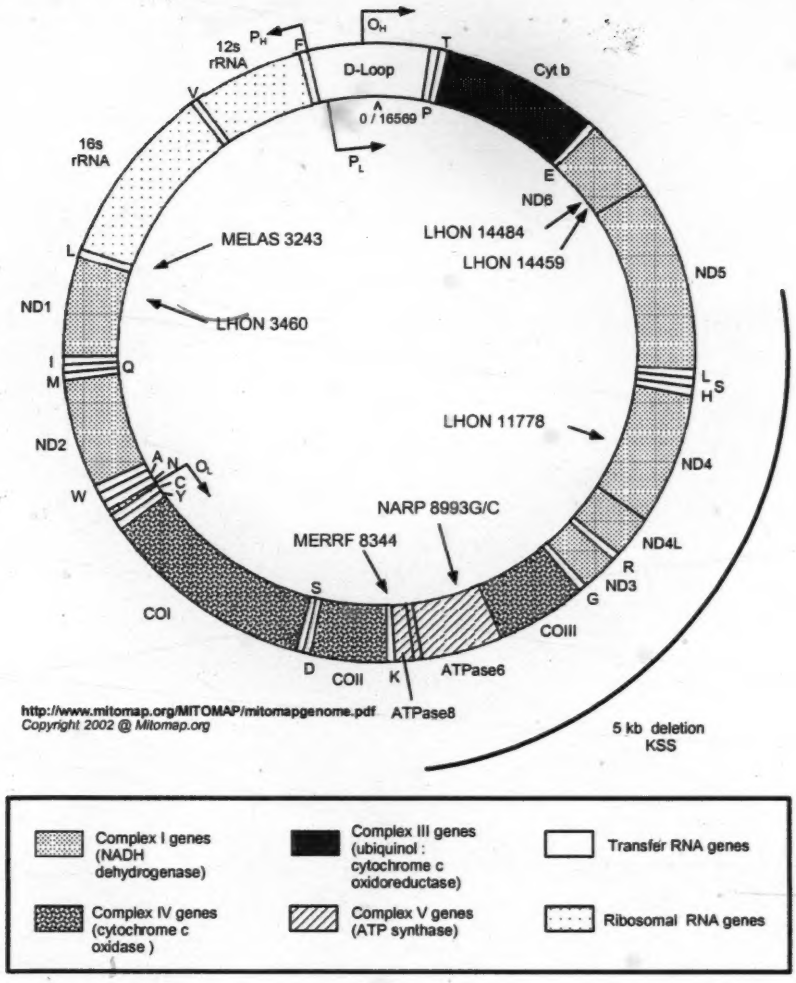

Figure 1.2 Human Mitochondrial Genome Map

The human mtDNA map, showing the different shaded areas representing the protein coding genes for seven subunits of complex I, one subunit of complex III (cytochrome *b*), three subunits of complex IV, two subunits of complex V (ATPase 6 and 8), ribosomal RNA (12s and 16s), and the 22 transfer RNAs represented by the one letter amino acid symbol. The heavy (H) strand origin of replication (O_H) and the light (L) strand promoters, P_H and P_L are illustrated in the control region. The locations of the pathogenic point mutations are shown on the inside of the circle, with the disease acronym and nucleotide location. The most common deletion area is shown by the external arc.

(Robin and Wong 1988; Shuster et al. 1988). Each human cell contains hundreds of mitochondria and thousands of mtDNA molecules.

Due to the large number of mtDNA molecules in each cell, when a mutation occurs in the cellular mtDNA, it can create a mixture of both normal and mutant mtDNA in the intracellular population. Cells that contain one mtDNA sequence are homoplasmic, whereas those with more than one mtDNA sequence are heteroplasmic (Shoffner 2001). The amount of mutated mtDNA can drift toward pure mutant or normal — a process referred to as replicative segregation. Previously, this process was thought to occur over generations, but segregation of mtDNA occurs by random genetic drift in early oogenesis in the small population of mtDNA in the precursors of primary oocytes (Jenuth et al. 1996; Lightowlers et al. 1997). Generally, individuals who do not have an OXPHOS disease are homoplasmic, but various studies have shown that the rate of heteroplasmy in somatic cells may be more frequent than originally hypothesized (Lightowlers et al. 1997). Each time a heteroplasmic cell divides, the normal and mutant DNA randomly segregate in the daughter cells. This replicative segregation results in great variation in both cellular genotypes and clinical phenotypes.

The mtDNA has a 10 to 20 times higher mutation rate than nuclear DNA (Johns 1995). Unlike nuclear DNA, the mtDNA has no introns, so a random mutation will generally affect a DNA coding sequence. The lack of

protective histones, poor repair mechanisms and a high number of replications per cell cycle also contribute to the high mutation rate.

The innate complexity of OXPHOS genetics is increased by several other phenomena of mitochondrial genetics that become apparent only when an OXPHOS disorder is present. These phenomena include the threshold expression of phenotypes, tissue-specific and developmental-stage gene expression and the accumulation of somatic mtDNA mutations with aging.

The severity of the OXPHOS defect resulting from mutated mtDNA is a function of the proportion of the mutant mtDNA and the differing energy requirements of the various organs and tissues (Wallace 1992a). This is referred to as threshold expression and is reflected by the reliance of each organ or tissue on mitochondrial energy production. When the mutant mtDNA accumulates, there is no apparent clinical phenotype until the proportion of mutant to normal mtDNA reaches a theoretical threshold. The biological behaviour of the cell will then change, reflecting an impaired energy state (Jackson et al. 1995).

The amount of OXPHOS activity utilized for energy production varies among tissues, cell types and developmental stages (Shoffner 2001). The nuclear location of many OXPHOS genes allows for tissue-specific regulation of energy metabolism. Single copy OXPHOS genes can show variable expression in some tissues. The ATP synthase β subunit, for example, is expressed at higher levels in heart and muscle

than in other tissues (Neckelmann et al. 1989). Genetic regulation of expression of both nuclear DNA and mtDNA OXPHOS genes varies during development, cell growth and neoplastic transformation (Webster et al. 1990). Some of the differences in OXPHOS activity between different tissues at various developmental stages are due in part to regulation of nuclear genes at the transcriptional level (Neckelmann et al. 1989; Webster et al. 1990).

The final feature bearing on OXPHOS genetics is the degenerative changes in proteins, lipids, nuclear DNA and mtDNA of the cell that occur with aging. These changes can result in cellular dysfunction and ultimately in cell death. Free radicals such as superoxide anion and hydrogen peroxide are produced continuously in the mitochondria by OXPHOS (Ames et al. 1995; Ozawa 1995). Although 96% to 99% of the oxygen delivered to the cell is reduced to water by cytochrome *c* oxidase, approximately 1% to 4% of the oxygen can be converted to oxygen radicals (Wallace 1992b). This occurs by direct transfer of electrons from reduced mitochondrial flavins, ubiquinone and cytochrome *b* to oxygen, thus producing oxygen radicals. A defect in the respiratory chain stimulates free radical formation and oxidative damage to mtDNA has been attributed to the close location of the DNA near the mitochondrial membrane where oxidants are formed (Ames et al. 1995). Oxidative degeneration of mitochondrial lipids, proteins and mtDNA impairs OXPHOS efficiency and stimulates more free radical damage, which

becomes a self-perpetuating process (Ames et al. 1995). The accumulation of somatic mtDNA mutations may contribute to the progression of mitochondrial diseases and account for the age-related decline in OXPHOS found in many neurodegenerative conditions.

In addition to mutations in either genome, defects of intergenomic communication may also cause an OXPHOS disorder. An increasing number of Mendelian disorders have also been identified that can be described as mitochondrial diseases, but the exact effect on OXPHOS is not known. Classification of these disorders is further complicated by the diversity of clinical symptoms, ranging from neurological or neuromuscular symptoms to non-neurologic symptoms resulting from involvement of organs such as liver, heart, kidney, bone marrow, pancreas, or gastrointestinal tract (De Vivo 1993).

OXPHOS diseases are divided into two categories: nuclear DNA mutations and mtDNA mutations. Mutations in nuclear OXPHOS genes are the most common causes for OXPHOS diseases, especially in the pediatric population (van den Heuvel and Smeitink 2001). The majority of the nuclear mutations produce a severe and fatal disease in infants and are inherited in an autosomal recessive manner (Shoubridge 2001). Adult-onset Mendelian OXPHOS disorders, which can be inherited in an autosomal or dominant pattern, generally have a milder phenotype and most frequently are caused by mtDNA deletions. Over the past 14 years, more than 100 mutations have been discovered in mtDNA

(Mitomap:http://www.gen.emory.edu/mitomap.html); these disorders are generally found in the adult population. Although biochemical classification has inherent problems (because many of the mitochondrial disorders affect multiple respiratory enzymes), in the absence of genetic information, OXPHOS disorders may also be described according to the individual respiratory chain complex that is defective, or according to the genetic mutation identified.

Nuclear OXPHOS Gene Mutations

Nuclear gene mutations causing disease are divided into two categories: **(1)** mutations in respiratory chain subunits, and **(2)** mutations in genes affecting assembly and maintenance proteins (van den Heuvel and Smeitink 2001).

(1) Complex I is the largest complex of the OXPHOS enzymes, and defects of complex I are likely the most common form of respiratory chain disease (De Vivo 1993). To date, mutations in five nuclear-encoded complex I structural subunit genes have been identified. In a patient with encephalopathy, a mutation causing a tandem five base pair (bp) duplication was identified in the NDUFS4 gene (van den Heuvel et al. 1998). A truncating mutation in the same gene was found in two patients with Leigh syndrome (LS), which is characterized by symmetrical focal necrotic lesions of the brain and brainstem (Budde et al. 2000). This latter mutation also caused a mild complex III defect in addition to the complex I deficiency. It is hypothesized that the defect in complex I alters the

structural integrity of the OXPHOS system, leading to the observed biochemical changes in complex III activity (Budde et al. 2000). Missense mutations in the NDUFS7 and NDUFS8 genes have also been found in other LS patients (Loeffen et al. 1998; Triepels et al. 2000). In two patients presenting with leukodystropy and myoclonic seizures, mutations were found in the NDUFV1 gene (Schuelke et al. 1999). Mutations in the NDUFS2 gene have been discovered in patients with encephalopathy and hypertrophic cardiomyopathy (Loeffen et al. 2001). To date, mutations in the nuclear-encoded subunits have only been found in approximately 35% of the patients with an isolated complex I deficiency, due to the vast number of nuclear genes dispersed throughout all the chromosomes affecting complex I activity (van den Heuvel and Smeitink 2001).

Complex II is the only respiratory chain complex in which the structural subunits are entirely encoded by nuclear genes. Mutations in the succinate dehydrogenase gene subunit have been found in a family with a late-onset neurodegenerative disease (Birch-Machin et al. 2000) and in two families with LS (Bourgeron et al. 1995; Parfait et al. 2000). In families with autosomal dominant hereditary paraganglioma, a disorder characterized by benign, highly vascularized tumors of the parasympathetic ganglia, mutations have been identified in the cytochrome *b* subunits (Baysal et al. 2000; Niemann and Muller 2000). It is speculated that complex II may act as a cellular oxygen sensor, because the pathology of the paragangliomas resembles that seen in the

carotid body under conditions of hypoxia (Baysal et al. 2000). Why some mutations appear to be cancer-susceptibility genes and others cause LS is unknown.

(2) Assembly gene defects have been associated with various mitochondrial diseases. Defects in nuclear genes encoding proteins that play important roles in assembly, import or stabilization of complex subunits may cause dysfunction of respiratory chain enzymes. A defect in the assembly of the NDUFS4 subunit in complex I has been identified in a patient with LS (Petruzzella et al. 2001). In complex III, BCS1L (the gene whose product is involved in assembly of complex III) has been found to be mutated in four Turkish families presenting with hepatopathy, encephalopathy, and tubulopathy (de Lonlay et al. 2001). The mutation appears to be tissue-specific, because the assembly defect could not be detected in fibroblasts (de Lonlay et al. 2001).

Complex IV deficiency, inherited in an autosomal recessive pattern, can present with a wide range of clinical phenotypes. The phenotypes include a classical LS, a French-Canadian form of LS, a fatal infantile COX deficiency, hypertrophic cardiomyopathy and myopathy, and a reversible COX deficiency confined to skeletal muscle (Robinson 2000). Evaluations of skin fibroblasts obtained from patients with COX deficiency have indicated that there was defective assembly of the enzyme in all cases, with the amounts of the nuclear and mitochondrial-encoded subunits reduced (Glerum et al. 1988). Presently, no mutations have been

identified in any of the nuclear-encoded structural genes of complex IV. SURF1, localized to chromosome 9q34, was the first nuclear COX gene shown to be mutated in COX deficiency (Tiranti et al. 1998). Mutations in SURF1 are generally associated with a LS phenotype; patients demonstrate an accumulation of an early assembly intermediate of the COX complex (Tiranti et al. 1998). Fatal, early onset, hypertrophic cardiomyopathy with encephalopathy has been associated with mutations in SCO2, a gene encoding a mitochondrial protein involved in supplying the COX enzyme with copper (Papadopoulou et al. 1999; Jaksch et al. 2000). Mutations in SCO1, a possible copper transfer protein, have been reported in a family with hepatic failure and ketoacidotic coma (Horvath et al. 2000). Mutations in COX10, a gene which encodes the heme A farnesyltranferase, have been identified in a patient with leukodystrophy and proximal tubulopathy (Valnot et al. 2000).

A patient (with cardiomyopathy, hepatomegaly and lactic acidosis) with a clear assembly defect of Complex V has been described, but in this case the gene defect has not yet been identified (Houstek et al. 1999). Although knowledge concerning the nuclear genes encoding OXPHOS complexes has expanded greatly over the past few years, the molecular basis for the majority of the autosomal recessive OXPHOS disorders remains unknown.

Mitochondrial DNA OXPHOS Mutations

While nuclear gene mutations have been separated into two general classes, the mtDNA mutations are divided into three classes: **(1)** mtDNA rearrangements in which mtDNA genes are deleted or duplicated; **(2)** mtDNA point mutations in tRNA or rRNA genes, resulting in mitochondrial protein synthesis defects; and **(3)** missense mutations that change an amino acid, causing an alteration in the structure or function of an OXPHOS polypeptide.

(1) Mitochondrial DNA deletions and duplications can be either maternally transmitted or occur as a sporadic event. The most common causes for Kearns-Sayre syndrome (KSS) and chronic progressive external ophthalmoplegia (CPEO) are mtDNA rearrangements which consist of mtDNA deletion mutations and mtDNA duplication mutations (Holt et al. 1988). It is estimated that 83% of KSS and 47% of CPEO cases are the result of mtDNA rearrangements (Moraes et al. 1989). In the majority of patients with mtDNA rearrangements, the mutation appears to be a spontaneous event that occurs at the time of fertilization of the oocyte (Marzuki et al. 1997). The most common mtDNA rearrangement is a 4977 base pair (bp) deletion that is flanked by two 13 bp direct repeats at nucleotides 8469 and 13447 (Schon et al. 1989) (Figure 2). This region extends from the ATPase 8 gene to the ND5 gene and affects the functioning of complexes I, III, IV, and V (Shoffner et al. 1989). Two other frequently observed rearrangements remove approximately five kilobases

(Kb) and seven Kb of mtDNA respectively. It is not possible to predict the clinical phenotype from the size or location of the mtDNA deletion. The same five Kb deletion has been identified in patients with KSS, CPEO, and Pearson syndrome (Fischel-Ghodsian et al. 1992). Pearson syndrome predominantly affects the bone marrow and manifests as a severe macrocytic anemia with neutropenia and thrombocytopenia (Pearson et al. 1979). Patients often die by age ten due to complications of bone marrow failure, or may survive and go on to develop symptoms of KSS in other organs later in life. KSS generally has an onset before 20 years, and is characterized by ophthalmoplegia, retinitis pigmentosa, and mitochondrial myopathy; it may also be associated with cardiac conduction defects, cerebellar ataxia, or a cerebrospinal fluid (CSF) lactate over 100 mg/dl (Shoffner 2001). Patients are classified as having CPEO if the clinical symptoms occur after 20 years; the phenotype consists of isolated extraocular eye muscle involvement to more complicated clinical manifestations. Patients with additional clinical symptoms are often referred to as CPEO PLUS patients. These symptoms can include optic atrophy, hearing loss, dementia, mitochondrial myopathy, diabetes, respiratory failure, neuropathies and ataxia (Moraes et al. 1989). Tissue distribution of the mutated mtDNA may be a critical determinant of phenotype. Unlike patients with Pearson syndrome, where the deletion is detectable in leukocytes, in KSS and CPEO patients the deletion is generally only found in post-mitotic tissue such as muscle. It is predicted

that high proportions of deleted mtDNA inhibit cell replication; as a result, most bone marrow cells affected by the deletion stop replicating and only normal bone marrow cells survive (Wallace et al. 2001).

(2) The majority of maternally inherited mitochondrial disorders are due to mtDNA point mutations; these mutations frequently affect mitochondrial protein synthesis. Myoclonic epilepsy with ragged red fibers (MERRF) is characterized by progressive myoclonic epilepsy, mitochondrial myopathy, and slowly progressive dementia (Wallace et al. 1988). Clinical features may also include optic atrophy, hearing loss, short stature and neuropathy (Silvestri et al. 1993). The ragged red fibers are visualized by the use of a modified Gomori-trichrome stain and appear as purplish-red irregular patches of abnormal mitochondria around the periphery of the muscle fiber (Taylor and Turnbull 1997). Although considered a hallmark of the disease, some individuals with the MERFF mutation do not show the proliferation of abnormal mitochondria (ragged-red fibers) on muscle biopsy (Taylor and Turnbull 1997). MERFF is most commonly caused by an A to G transition at nucleotide 8344 in the $tRNA^{Lys}$ gene (Shoffner et al. 1990). It has been shown that the mutated tRNAs cause a severe reduction in mitochondrial protein synthesis and produce aberrant translation products of unknown functional significance (Enriquez et al. 1995). Biochemical analysis of muscle tissue from MERFF patients has demonstrated a combined complex I and IV deficiency (Wallace et al. 1988).

Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) is a progressive neurodegenerative disease characterized by the symptoms described in the acronym. Additional symptoms may include seizures, recurrent vomiting, migraine-like headaches, limb weakness, exercise intolerance, hypertrophic cardiomyopathy, ophthalomoplegia, pigmentary retinopathy, dementia, deafness, type II diabetes, and short stature (Ciafaloni et al. 1992; Hirano and Pavlakis 1994). As suggested by the spectrum of symptoms, the clinical phenotype varies from a severe multisystemic disorder starting in infancy to patients presenting late in life with focal neurological deficits (Koo et al. 1993). Approximately 80% of individuals with clinical features of MELAS have a heteroplasmic A to G point mutation in the dihydrouridine loop of the $tRNA^{Leu}$ gene at bp 3243 (Goto et al. 1990; Enter et al. 1991; Ciafaloni et al. 1992). Another 7.5% have a heteroplasmic T to C mutation at bp 3271 in the $tRNA^{Leu}$ gene (Goto et al. 1991; Tokunaga et al. 1993). Unidentified mtDNA mutations are thought to be the cause of the remaining cases of MELAS. Although our understanding of the biochemistry underlying this disease is incomplete, decreased complex I activity is the most frequent biochemical finding associated with MELAS (Hirano and Pavlakis 1994). Complex III and IV deficiencies alone, or in combination with a complex I deficiency, have also been observed in studies of MELAS patients (Koo et al. 1993).

(3) Mutations in mtDNA protein-coding genes include Leber's hereditary optic neuropathy (LHON) and neurogenic ataxia and retinitis pigmentosa (NARP). LHON is a maternally inherited disease characterized by acute central vision loss, usually in young adults and predominantly in males (Wallace et al. 2001). In a small percentage of cases, patients may develop clinical or radiological signs similar to multiple sclerosis (Nikoskelainen et al. 1995; Olsen et al. 1995). LHON is associated with three main mutations in complex I: G11788A in ND4, G3460A in ND1, and T14484A in ND4 (Brown 1999). In most cases of mtDNA disorders, the mutation is heteroplasmic; the exception is LHON, in which the mutation is frequently found in the homoplasmic state (Brown 1999). Studies conducted on the biochemical defect associated with the LHON mutation have yielded conflicting results. Complex I activity has been shown to be reduced from 0% to 50% relative to control values, but most reports do not demonstrate a statistical significant reduction (Larsson et al. 1991; Majander et al. 1991; Degli Esposti et al. 1994; Smith et al. 1994; Carelli et al. 1997). NARP is associated primarily with the T8993G mutation in the ATPase 6 gene (Holt et al. 1990). The T to C point mutation at the same position has also resulted in similar clinical symptoms (de Vries et al. 1993). Clinical features include pigmentary retinal changes, cerebellar ataxia, seizures, cognitive impairment and peripheral neuropathy (Holt et al. 1990). Some patients with this mutation have been described as suffering from maternally inherited Leigh syndrome (MILS). Biochemical

analysis of lymphoblast mitochondria from patients with the NARP mutation has shown decreased ATP synthesis with all substrates tested (Tatuch and Robinson 1993).

Access to automated sequencing technologies has allowed the determination of the complete mitochondrial genome in patients and has made possible the identification of many mtDNA mutations. The above descriptions only highlight those mutations most commonly encountered in clinical practice.

Nuclear-Mitochondrial Communication Disorders

Defects of intergenomic communication can affect the integrity of the mitochondrial genome resulting in qualitative alterations of mtDNA, and quantitative decrease of mtDNA copy number (Clayton 1998). Diseases caused by nuclear genes that affect mtDNA stability and maintenance demonstrate accumulation of multiple large-scale mtDNA deletions (Suomalainen and Kaukonen 2001). They are inherited in a Mendelian manner, indicating that a nuclear gene defect causes the mtDNA mutation or depletion (Zeviani et al. 1997). Multiple mtDNA deletions have been found in families with a form of CPEO which is inherited in an autosomal dominant fashion (Zeviani et al. 1989; Suomalainen et al. 1992). Southern blot analysis of muscle mtDNA from these patients revealed the presence of different deletions among the mtDNA examined (Zeviani et al. 1989). Mutations in the genes encoding adenine nucleotide translocator 1 (ANT), Twinkle, a mitochondrial helicase and polymerase gamma (POLG) have

also been identified as causes of autosomal dominant PEO (Kaukonen et al. 2000; Spelbrink et al. 2001; Van Goethem et al. 2001). Clinical features include adult onset external ophthalmoplegia, proximal weakness and wasting, and sensorineural hearing loss. Complex I and IV activity in patient muscle samples ranged from normal to 50% of the control value (Zeviani et al. 1989). An autosomal recessive variant of CPEO with multiple mtDNA deletions has also been reported (Mizusawa et al. 1988), and a phenotype of recurrent exertional myoglobinuria and rhabdomyolysis has been described in two brothers, with the subsequent finding of deleted mtDNA in muscle (Ohno et al. 1991).

Two further syndromes involving dysfunction in intergenomic communication have been described. Depletion of total mtDNA is thought to be a result of a mutation in an unidentified nuclear gene controlling mtDNA copy number (Moraes et al. 1991; Tritschler et al. 1992). Generally, the clinical phenotype ranges from a fatal infantile myopathy to a childhood myopathy characterized by respiratory failure and death by age three. Recently, eight families with a late onset and slowly progressive disease have been reported (Barthelemy et al. 2001). Mitochondrial neurogastrointestinal disorder and encephalopathy (MNGIE) is an autosomal recessive disorder presenting with external ophthalmoplegia, leukodystrophy, mitochondrial myopathy, peripheral neuropathy and prominent involvement of the gastrointestinal tract (Bardosi et al. 1987). This disorder is caused by loss-of-function mutations in the thymidine

phosphorylase gene, the role of which is to regulate thymidine availability for both mitochondrial and nuclear DNA synthesis (Nishino et al. 1999).

Indirect Effects on OXPHOS

A number of disorders that have autosomal inheritance are classified as mitochondrial diseases, although the effects of these mutations on oxidative phosphorylation are unclear. Mohr-Tranebjerg syndrome is an X-linked recessive disorder caused by mutations of the dystonia deafness polypeptide (DDP), a gene encoding the human homologue of Tim8, (translocase of the inner membrane), a yeast protein that mediates the import of mitochondrial proteins. The disorder is characterized by deafness, dystonia, optic atrophy and dementia (Koehler et al. 1999). The respiratory chain is affected in Freidreich's ataxia, which is due to a GAA trinucleotide repeat in the frataxin gene. Frataxin gene mutations result in mitochondrial iron overload, which causes impaired activity of the iron-sulfur containing enzymes in complexes I, II, and III (Campuzano et al. 1996). Clinical symptoms include ataxia, absent deep tendon reflexes and hypertrophic cardiomyopathy. Hereditary spastic paraplegia is a genetically heterogeneous group of neurodegenerative disorders characterized by progressive weakness and spasticity of the lower limbs. In certain families, the disease is caused by mutations in the paraplegin gene (localized to chromosome 16q24), the function of which is related to turnover of mitochondrial inner membrane proteins (Casari et al. 1998). Dominant optic atrophy is due to mutations in the OPA1 gene; its protein

product is thought to be involved in the control of mitochondrial morphology (Delettre et al. 2000).

DIAGNOSTIC EVALUATION IN OXPHOS DISORDERS

Metabolic Investigations

Metabolic evaluation is often the first approach when screening patients with suspected OXPHOS disorders. A frequent indication of respiratory chain dysfunction is an abnormal redox state, detected by abnormal lactate and/or pyruvate levels. This is partially due to the functional impairment of the citric acid cycle, caused by a defect in the respiratory chain which leads to an excess of NADH and a lack of NAD (Horton et al. 1996). This in turn causes a secondary elevation in lactate and increased lactate/pyruvate ratio. A number of other disruptions in intermediary metabolism have been noted to result from respiratory chain dysfunction. Pyruvate may be transferred to the cytosol as alanine, causing hyperalaninemia (Horton et al. 1996). Inhibition of fatty acid oxidation may also occur, as acyl CoA dehydrogenase is involved in transferring electrons to electron-transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase. The 3-hydroxyacyl CoA dehydrogenase reaction involves the reduction of NAD to NADH and is inhibited by elevated concentrations of NADH, such as those caused by an OXPHOS defect (Adams and Turnbull 1996). An increased ratio of blood lactate to pyruvate (L/P), accompanied by an increased ratio of 3-hydroxybutyrate to acetoacetate (>2:1), is highly suggestive of an OXPHOS defect (Robinson 2001). In

contrast, elevated plasma lactate with an elevated L/P ratio and low ketone ratio may be indicative of a Kreb's cycle disorder or pyruvate carboxylase deficiency, while elevated lactate with a low L/P ratio may indicate a pyruvate dehydrogenase deficiency (Robinson 2001). Pitfalls in metabolic screening are numerous and include: (1) an artificial elevation of lactic acid due to improper collection and processing of the blood sample; (2) proximal renal tubulopathy may lower blood lactate and increase urinary lactate; (3) an elevated lactate may be latent in basal conditions and only revealed by exercise testing and glucose loading; and (4) the defect may be tissue-specific, barely altering the redox state in the plasma. The metabolic investigations are further complicated by the fact that a number of patients with OXPHOS defects present with a normal blood lactate (Jackson et al. 1995). It is important to point out that a negative result does not rule out an OXPHOS defect, and a positive result is only the starting point in identifying the defect. Newer methods of molecular analysis have allowed us to more clearly identify specific defects.

Molecular Testing

Certain "classical" presentations are highly suggestive of a known mitochondrial syndrome associated with specific mtDNA mutations. Well recognized syndromes that can generally be detected by current molecular analysis include MELAS, MERRF, LHON and NARP. These mutations can frequently be detected in leukocyte mtDNA with no further

diagnostic testing required (Shoffner 2001). Kearns-Sayre syndrome and CPEO often require molecular analysis to be performed on a post-mitotic tissue such as muscle to establish a definite diagnosis. Although the presentations of these disorders may be "classical," it must be emphasized that mtDNA diseases are phenotypically diverse and may present with non-typical features. Unfortunately, the majority of patients have a combination of clinical features suggestive of an OXPHOS defect, but lack a molecular genetic diagnosis. To confirm the diagnosis, a combination of pathological and biochemical analysis may be necessary.

Histochemical Evaluation

Histochemical analysis and electron microscopy of muscle biopsy tissue may be helpful in supporting the diagnosis of an OXPHOS defect. The ragged red fiber (RRF) detected by the modified Gomori-trichrome stain is caused by a proliferation of subsarcolemmal mitochondria and is a histological hallmark of mitochondrial myopathies (Taylor and Turnbull 1997). Electron-microscopic evidence of abnormal mitochondria (including paracrystalline inclusions, giant mitochondria, or proliferation of normal mitochondria) may also be found in OXPHOS disorders (Walker et al. 1996). Although the diagnostic importance of pathological studies should not be discredited, the absence of RRF's and structural mitochondrial abnormalities does not rule out an OXPHOS defect. In addition, histochemical stains for oxidative enzymes are used to estimate enzyme activity in tissue sections. Methods are readily available for succinate

dehydrogenase (SDH) and cytochrome *c* oxidase (COX), but histochemical methods for detecting defects of complex I and III are not reliable (Adams and Turnbull 1996). Despite the progress made in histopathology, it is difficult to correlate genetic abnormalities with mitochondrial ultrastructure and cellular dysfunction.

Spectrophotometric Studies

Conclusive diagnostic evidence of respiratory chain deficiency is often provided by spectrophotometric studies. These studies measure respiratory enzyme activities separately or by enzyme groups, in isolated mitochondria or whole cell homogenates, using specific electron acceptors and donors (Munnich et al. 1996). Generally, spectrophotometric investigations have utilized the muscle biopsy as the tissue of choice. Cultured skin fibroblasts or lymphoblast cell lines can also be utilized for spectrophotometric studies. The use of cultured skin fibroblasts or lymphoblasts has some disadvantages, because of the concern about variability in expression in these cells due to tissue-specific respiratory chain defects. There is also the added problem of interactions between cell transformations and mitochondrial activity in transformed lymphocytes (Bourgeron et al. 1993). Some defects may not be detected in mitochondrial fractions isolated from cultured cells (Taylor and Turnbull 1997). In a patient with a complex I defect, the analysis of mitochondria isolated at autopsy showed great variability in residual enzyme activity with 2% in skeletal muscle, 11% in liver, 16% in heart, and 33% in kidney

(Moreadith et al. 1984). Complex I defects have been diagnosed in skin fibroblasts, but the reliability of assessing complex I activity in cultured cells is questionable due to the rotenone-resistant cellular NADH cytochrome *c* reductase activity (Munnich et al. 1996). Due to the high level of rotenone-resistant activities in other cell components, the complex I and III coupled assay must also be performed on mitochondrial fractions, not cell homogenates (Trounce et al. 1996). When the skeletal muscle clinically expresses the disease, muscle biopsy may be the most appropriate tissue for analysis. The disadvantages of muscle biopsy for mitochondrial isolation are that it is an invasive procedure, requires a substantial amount of tissue, and that artifacts can occur in this system, particularly in subjects who have abnormal lipid storage in muscle (Weber et al. 1997). The *in vitro* investigation of OXPHOS defects remains difficult regardless of the tissue tested.

Polarographic Studies

Polarographic studies measure the oxygen consumed by mitochondrial preparations using a closed chamber and a Clarke oxygen electrode (Estabrook 1967). When the mitochondrial preparation is suspended in a buffer (containing ADP, inorganic phosphate and a respiratory substrate), active respiration is carried out and the oxygen consumption reflects the activity of the respiratory chain enzymes (Trounce et al. 1996). Polarographic studies can be performed on mitochondrial-enriched fractions of skeletal muscle, intact circulating lymphocytes, or detergent-

permeabilized cultured cells such as skin fibroblasts or lymphoblast cell lines (Munnich et al. 1996). The limitations with polarographic studies are that the technique requires a large amount of mitochondrial protein to give reliable results, and the analysis must be performed on fresh material (Taylor and Turnbull 1997).

ATP Synthesis

OXPHOS is responsible for at least 90% of the total ATP produced in eukaryotic cells containing mitochondria. The remaining ATP synthesis occurs in the surrounding cytoplasm through glycolysis. A variety of methods can be employed to quantify ATP production from isolated mitochondria and permeabilized cells. One method utilizes $^{32}P_i$ incorporation into ADP and subsequent transfer into glucose-6-phosphate by hexokinase, followed by extraction of unincorporated $^{32}P_i$ and measurement of radioactivity (Tuena de Gomez-Puyou et al. 1984). An alternate method is based on the luciferin-luciferase system, where the bioluminescence properties have been used to measure ATP in isolated mitochondria (Wibom et al. 1990) and permeabilized cells (Ouhabi et al. 1998). Fluorimetric methods have been employed using cultured skin fibroblasts and cultured lymphoblasts to examine ATP synthesis (Bourgeron et al. 1992; Robinson 1996). Initially, due to the low permeability of the plasma membrane, assays were performed on isolated mitochondria, which required a substantial amount of tissue (Tuena de Gomez-Puyou et al. 1984; Tatuch and Robinson 1993). Membrane

permeabilization by detergents such as digitonin allows mitochondrial OXPHOS to be assessed indirectly on whole cells (Robinson et al. 1986). The use of respiratory substrates that enter the respiratory chain at different sites also imparts information about the enzyme complex that is affected. Regardless of the defect, the end result of respiratory chain dysfunction is a reduction the production of cellular ATP. The measurement of mitochondrial ATP synthesis can serve as an additional tool to assess cellular energy metabolism in mitochondrial disorders.

SUMMARY

The investigation of a patient with a suspected OXPHOS defect generally requires a combination of clinical, molecular and biochemical studies to define the precise nature of the defect. Much progress has been made in defining the molecular abnormalities in these patients, but in the majority of the cases the underlying genetic defect remains unresolved. Biochemical analysis remains an important aspect of the diagnostic investigation. Diagnostic screening methods are necessary to screen for OXPHOS disorders and to guide clinicians to further more invasive procedures. The development of a relatively non-invasive diagnostic method to screen for OXPHOS disorders is crucial. Because current information on the evaluation of effective treatment approaches is limited, this diagnostic tool would be instrumental in providing an objective measurement for monitoring response to treatment and evaluating progression of disease.

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CHAPTER 2

COFACTOR TREATMENT IN OXPHOS DISORDERS

INTRODUCTION

Therapeutic trials in OXPHOS disorders are difficult to conduct, because the diseases are rare and demonstrate vast clinical and genetic heterogeneity. Many reports of treatment have been anecdotal with relatively short follow-up. The unpredictable and variable natural history of these disorders and the lack of reliable clinical outcome measures also make it difficult to evaluate these reports. The goal of nutritional cofactor therapy is to increase mitochondrial ATP production and slow or arrest the progression of clinical symptoms. Accumulation of toxic metabolites and reduction of oxidative capacity have prompted the use of antioxidants, electron transfer mediators (which by-pass the defective site) and enzyme cofactors. Metabolic therapies that have been reported to produce a positive effect include coenzyme Q_{10} (ubiquinone), other antioxidants such as ascorbic acid and vitamin E, riboflavin, thiamine, niacin, vitamin K (phylloquinone and menadione) and carnitine. A literature review of the use of these supplements in the treatment of OXPHOS disorders is presented.

COENZYME Q_{10} (UBIQUINONE)

CoQ_{10} is the most widely used supplement in the treatment of mitochondrial disorders.Coenzyme Q_{10} is a fat-soluble quinone containing ten isoprenoid units; it transfers electrons from complexes I and II to

complex III, a process that is coupled to ATP synthesis (Rauchova et al. 1995). In its reduced form (ubiquinol), coenzyme Q_{10} also inhibits lipid peroxidation and can protect mitochondrial inner membrane proteins and DNA against oxidative damage (Ernster and Dallner 1995). Ubiquinol acts as an inhibitor of lipid peroxidation by preventing formation of lipid peroxyl radicals and regenerating vitamin E from the *α*-tocopheroxyl radical (Kagan et al. 1990). Coenzyme Q_{10} also has a function in stabilizing the OXPHOS complexes within the inner mitochondrial membrane by maintaining optimal membrane fluidity (Fato et al. 1984). Endogenous synthesis and dietary sources, primarily animal products, such as meat, fish and poultry, contribute to normal CoQ_{10} levels in plasma. The average daily intake of CoQ_{10} from the diet was estimated to be 3 to 5 mg per day in the Danish population (Weber et al. 1997). The optimal dietary requirement for CoQ_{10} is unknown. The contribution of dietary sources to CoQ_{10} levels in plasma, in comparison to the amount contributed through *de novo* synthesis is not known.

Coenzyme Q_{10} has been reported to have a beneficial effect on clinical outcome and biochemical parameters in a variety of OXPHOS disorders. The positive effects have included a reduction of cerebrospinal fluid (CSF) and serum lactate and pyruvate (Ogasahara et al. 1985; Goda et al. 1987; Yamamoto et al. 1987; Bresolin et al. 1988; Nishikawa et al. 1989; Abe et al. 1991), improvement in cardiac conduction defects and ocular movements (Ogasahara et al. 1985), reduced muscle weakness

(Yamamoto et al. 1987; Ihara et al. 1989; Abe et al. 1991), improved exercise tolerance (Goda et al. 1987; Bresolin et al. 1988), improved oxygen utilization during exercise as measured by tissue oximetry (Abe et al. 1999), decreased peripheral nerve damage (Ihara et al. 1989), improvement in neurological function (Bresolin et al. 1988), increased respiratory chain activity (Schneider et al. 1982; Bresolin et al. 1988) and acceleration of post-exercise recovery detected by nuclear magnetic resonance (^{31}P-NMR) spectroscopy (Nishikawa et al. 1989; Bendahan et al. 1992). Most reports regarding treatment have been case studies or anecdotal reports with limited patient numbers, variable treatment periods, and with CoQ_{10} dosages ranging from 30 to 300 mg/day.

Several short-term studies have shown variable results with CoQ_{10} treatment. Two double-blind placebo trials with 12 and 15 patients respectively, used 100 mg/day of CoQ_{10} for three months in muscular dystrophies and neurogenic atrophies, (diseases included Duchenne, Becker and the limb-girdle dystrophies, myotonic dystrophy, Charcot-Marie-Tooth and Welander disease). The patients showed an improvement in cardiac function and physical performance (Folkers and Simonsen 1995). In another short-term (three months 160 mg/day CoQ_{10}, one month placebo), double-blind crossover study in eight patients with mitochondrial encephalomyopathies, a trend of effectiveness of CoQ_{10} was noted by improved muscle endurance, decreased fatigability of daily activities and decreased serum lactate and pyruvate levels, but statistical

significance was only noted in global muscle strength (Chen et al. 1997). In both of these studies, the authors questioned whether the dosages of 100 and 160 mg/day, respectively, were too low and whether the short-term administration was adequate. Clinical features of the 3243 mtDNA mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) mutation may also include neurodegenerative changes and diabetes. In an open trial of 11 patients with the MELAS 3243 mutation (treated for three to five months with CoQ_{10} dosages ranging from 30 to 210 mg/day), there was an improvement in neuromuscular symptoms, but no difference in fasting glucose or glycemic control (Andersen et al. 1997).

Longer-term studies of six-month duration have evaluated the effectiveness of CoQ_{10} treatment. A report of a six-month trial of 150 mg/day of CoQ_{10} in eight patients (using ^{31}P-NMR to objectively measure muscular function) indicated an improvement in the mean ratio of phosphocreatine (PCr) to inorganic phosphate (P_i), but the beneficial effect was mainly due to a single individual, and did not reflect a beneficial effect on the whole group (Gold et al. 1996). The phosphorylation potential (PCr/P_i ratio) at rest is lower, and there is a delay in post-exercise PCr/P_i recovery in mitochondrial patients. NMR measurements are considered a highly sensitive measurement of mitochondrial function. Phosphorous magnetic resonance spectroscopy (^{31}PMRS) was utilized to study the effect of six months of CoQ_{10} treatment on brain and skeletal muscle mitochondrial function in six patients. Baseline brain and skeletal muscle

metabolism was compared to 36 age-matched healthy controls. Mitochondrial function (represented by phosphorylation potential) in both brain and muscle was reduced by 25% and 29%, respectively, compared to controls. Treatment with CoQ_{10} statistically improved phosphorylation potential and calculated ATP synthesis in both brain and skeletal muscle in all patients studied (Barbiroli et al. 1997).

In a multi-center double-blind study of CoQ_{10} administration of 2 mg/kg/day for six months in 44 patients with mitochondrial myopathies, 16 subjects showed a 25% decrease in post-exercise lactate levels (Bresolin et al. 1990). Patients who responded were treated for an additional three months with CoQ_{10} or a placebo; no significant differences were detected between the two groups. A bicycle ergometry study in nine patients with mitochondrial encephalomyopathies, treated with 150 mg/day CoQ_{10}, showed no change in metabolic parameters after three months of therapy. After six months of treatment, four patients showed a decrease in lactate/pyruvate ratios at rest and in association with exercise (Chan et al. 1998). It is unclear why some patients respond while others with the same clinical phenotype and biochemical defect do not show any benefit. Response to treatment was not related to the CoQ_{10} level in serum or in platelet mitochondria, or to the type of molecular defect (Bresolin et al. 1990). Characterization of responders would contribute greatly to our understanding of the means by which CoQ_{10} exerts its beneficial effect.

The longest-term study examined supplementation of 150 mg/day of CoQ_{10} over a three-year period in MELAS patients with the 3243 mutation. Insulin secretory response, progression of hearing loss and lactate response after exercise were evaluated. In the 44 MELAS patients, 28 had diabetes and hearing deficits, 28 had impaired glucose tolerance, and 15 had normal glucose tolerance. The group of 28 patients with diabetes experienced an increased insulin secretory response and improved lactate response after exercise with the CoQ_{10} treatment. In addition, there was no further progression of hearing loss. The CoQ_{10} treatment did not affect the insulin secretory response of the patients with impaired and normal glucose tolerance. Short-term CoQ_{10} treatment (three months) in this study did not affect the insulin secretory response or clinical symptoms in any of the subjects (Suzuki et al. 1998).

While most cases of decreased CoQ_{10} levels are secondary to other causes, primary muscle CoQ_{10} deficiency has been documented in patients with a mitochondrial encephalomyopathy characterized by ragged-red fibers and lipid storage in muscle, recurrent myoglobinuria, seizures, ataxia and mental retardation (Sobreira et al. 1997; Rotig et al. 2000; Musumeci et al. 2001). In six patients with muscle CoQ_{10} deficiency, high doses of CoQ_{10} (300 to 3000 mg/day) resulted in dramatic improvement in strength, seizure control and ataxia (Musumeci et al. 2001). In another case report of CoQ_{10} deficiency, two patients were supplemented with 200 to 300 mg/day; after two months of therapy all

symptoms and muscle weakness resolved. Lactic acid levels and creatine kinase values normalized and remained normal during a three-year follow-up study. The activities of the respiratory chain enzymes increased two-fold and CoQ_{10} levels in muscle returned to normal. Histochemical evaluation examined apoptotic features before and after treatment and found that apoptotic features were markedly decreased in muscle tissue after CoQ_{10} supplementation. The authors conclude that CoQ_{10} has an important role in inhibiting apoptosis (Di Giovanni et al. 2001).

Controversy exists regarding the uptake of orally administered ubiquinone in various human tissues. It is well documented that serum and plasma levels of CoQ_{10} increase with supplementation. Animal and human studies have demonstrated that approximately 2% to 10% of the oral dose administered is taken up into the blood (Zhang et al. 1995; Weber 2001). Dosages of 90 to 150 mg/day of CoQ_{10} have been shown to increase plasma concentrations by 180% (Kaikkonen et al. 1997). It has also been demonstrated that CoQ_{10} levels increase in platelet mitochondria (Bresolin et al. 1988). In CoQ_{10} deficiency, oral supplementation resulted in an increase in CoQ_{10} in muscle (Rotig et al. 2000). Another study did not find an increase in muscle CoQ_{10} content with administration of 50 to 100 mg/day (Zierz et al. 1990). Animal studies which young rats (one to two month-old) were supplemented with 200 mg/kg of CoQ_{10} for one to two months resulted in a significant increase in liver concentration, but no significant increase in brain concentration (Beal

and Matthews 1997). The same authors fed 12 to 24 month-old rats the same dosage (200 mg/kg) of CoQ_{10} and observed a significant increase in the CoQ_{10} content of the cerebral cortex (Matthews et al. 1998). This finding suggests that CoQ_{10} levels in young animals may be tightly regulated and that the levels in membranes could be saturated. The increase in brain CoQ_{10} levels in the older animals lends support to the prospect that CoQ_{10} may be beneficial in the neurological manifestations of respiratory chain disorders.

Commercially prepared CoQ_{10} supplements are available as powder-filled hard shell capsules, oil-based suspensions in a soft gel capsule and emulsions in a soft gel capsule. There are limited reports on the bioavailability or absorption of CoQ_{10} in these preparations. Most studies have demonstrated that compounds formulated in soft gelatin capsules representing liquid suspensions tend to be absorbed more effectively than a dry powder blend encapsulated in hard gelatin capsules (Folkers et al. 1994; Weis et al. 1994; Wahlqvist et al. 1998). Similar serum CoQ_{10} levels have been obtained with emulsified and oil-based preparations (Lyon et al. 2001). Another report did not find any difference in bioavailability between an oil-based suspension or a granule preparation and observed that bioavailability varied more between subjects than between the two preparations (Kaikkonen et al. 1997).

The efficacy of CoQ_{10} supplementation in mitochondrial disorders is unclear. Many patients report improvement in clinical symptoms and side

effects from large pharmaceutical dosages are extremely rare. Dosage in the treatment of mitochondrial disorders varies, but clinicians currently recommend 4 to 15 mg/kg/day to determine efficacy in an individual patient (Gold and Cohen 2001). Additional studies are necessary to evaluate the therapeutic efficacy of CoQ_{10} in a variety of mitochondrial disorders.

ANTIOXIDANTS

Vitamin C

Vitamin C has been utilized in OXPHOS disorders for its antioxidant properties (Przyrembel 1987). Ascorbic acid acts as a reducing agent; the prevention of oxygen radical damage is the rationale for ascorbate administration. Respiratory chain enzyme activity has been evaluated in 10 to 20 week-old cultured fibroblasts with or without ascorbate treatment. Aging was associated with a decrease in respiratory chain activity and ascorbate significantly reduced the decrease in activity (Sharma et al. 1998). Ascorbate has been administered in combination with vitamin K_3 (menadione) to donate electrons directly to cytochrome *c* in a patient with complex III deficiency (Eleff et al. 1984). Marked improvement in recovery from exercise as measured by NMR spectroscopy has prompted clinicians to use this regime in other patients with OXPHOS disorders.

Antioxidants such as vitamin C may slow the process of oxidative damage, but the benefits over time are impossible to measure. Dosages typically range from 250 to 4000 mg/day (Gold and Cohen 2001). Adverse

effects such as diarrhea, although rare, have been attributed to several large doses (1 g/day) given throughout the day.

Vitamin E

The main function of vitamin E is to scavenge free radicals and inhibit lipid peroxidation, which helps maintain membrane integrity (Groff et al. 1995). Vitamin E includes eight compounds, with α-tocopherol having the greatest biological activity. The α-tocopherol/cholesterol ratio has been shown to be reduced in patients and asymptomatic carriers of the 11778 Leber's hereditary optic neuropathy (LHON) mutation (Klivenyi et al. 2001). The authors conclude that the impaired function of complex I increases free radical formation and that the reduced ratio of α-tocopherol/cholesterol reflects α-tocopherol consumption in the affected tissues. Addition of vitamin E to fluids surrounding cardiac muscle preparations has demonstrated protection against hypoxic trauma (Guarnieri et al. 1978). Radical scavenging antioxidants function independently, but may also act synergistically with other antioxidants. The tocopheroxyl radical formed is regenerated to active vitamin E by reaction with ubiquinol or ubisemiquionone (Kelso et al. 2002). Several studies indicate that α-tocopherol and CoQ_{10} are more efficacious when acting together (Kagan et al. 2000). In an animal study, CoQ_{10} administration resulted in an increase in both α-tocopherol and CoQ_{10} content in mitochondria, which was inversely correlated with a decrease of superoxide anion radical generation (Lass and Sohal 2000). Both

ubiquinol and ascorbate may play a role in the regeneration of active vitamin E (Kagan et al. 2000).

Vitamin E appears to be one of the least toxic vitamins. Although there are no proven benefits with vitamin E treatment in OXPHOS disorders, commonly used dosages are 400 to 1200 IU per day (Gold and Cohen 2001).

RIBOFLAVIN

Riboflavin is a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which function as cofactors in complexes I and II. Riboflavin has been reported to be effective in a number of patients with complex I deficiency (Arts et al. 1983; Griebel et al. 1990; Bernsen et al. 1991; Penn et al. 1992; Bernsen et al. 1993; Scholte et al. 1995; Ogle et al. 1997). It is postulated that riboflavin may act by inhibiting the breakdown of complex I by providing resistance to proteolysis, or by stabilizing the complex in the membrane, with subsequent increase in enzymatic activity (Vergani et al. 1999; Gold and Cohen 2001). Riboflavin-deficient rats show abnormalities in both biochemical and morphological aspects of mitochondria, adding support to this hypothesis (Addison and McCormick 1978).

Treatment solely with riboflavin has been utilized in patients with complex I deficiency. Improvement in exercise capacity after 100 mg/day of riboflavin has been noted in a patient with a NADH-CoQ reductase deficient myopathy (Arts et al. 1983). In a study of five patients with

complex I deficiency, definite clinical improvement was noted with riboflavin therapy in two patients with the myopathic form, while only one of the patients with the encephalomyopathic form improved (Bernsen et al. 1993). Complex I activity in three out of the five patients improved, but clinical improvement did not correlate well with the increase in enzyme activity (Bernsen et al. 1993). The results of the study were further complicated by various dosages of riboflavin (9 to 60 mg/day) and varied lengths of treatment. An infant with a partial complex I defect was treated with increasing doses of riboflavin (maximal 13 mg/kg/day or 120 mg/day). There was an improvement in motor development and normalization of lactate levels (Griebel et al. 1990). In fibroblasts from a patient with a nuclear-encoded complex I deficiency, the addition of 5μmol/L of riboflavin to the culture medium significantly increased ATP synthesis (Bar-Meir et al. 2001).

Riboflavin has been used for the treatment of respiratory chain disorders in combination with other cofactors. In a case study of a young boy with complex I deficiency, myopathy improved dramatically during treatment with riboflavin (9 mg/day) and carnitine (2 g/day). After seven months of treatment, complex I activity in muscle had normalized (Bernsen et al. 1991). Although fatty acid oxidation was normal, muscle carnitine levels before treatment were low and normalized after treatment. Further support for the therapeutic benefit of riboflavin alone or in combination with carnitine has been demonstrated in three adult family members with

complex I deficiency (Scholte et al 1995). Muscular endurance strength, as measured by bicycle ergometry, increased in all three subjects. One of the patients (receiving 300 mg riboflavin and 2 g carnitine daily), was found to have decreased resting lactate and increased PCr resynthesis, measured by ^{31}P-NMR. A muscle biopsy obtained from the second subject, after two years of 100 mg/day riboflavin supplementation, showed complex I activity increased from 16% to 47% of the mean control levels (Scholte et al. 1995). In a case report of a mitochondrial myopathy (due to a complex I deficiency) in a three-year-old female treated with both riboflavin (50 mg/day) and carnitine (100 mg/kg/day), clinical symptoms improved. The withdrawal of carnitine did not alter the clinical response (Ogle et al. 1997). Exercise tolerance deteriorated and muscle tone decreased when riboflavin was discontinued, thereby attributing the clinical improvement solely to riboflavin (Ogle et al. 1997).

A case study lending support to improvement in encephalomyopathic forms of OXPHOS disorders evaluated treatment of nicotinamide (1 g qid) and riboflavin (100 mg/day) in a patient with MELAS syndrome (Penn et al. 1992). To confirm clinical benefit, treatment was withdrawn; changes in ^{31}P-MRS and sural nerve conduction studies coincided with the development of encephalopathy (Penn et al. 1992). The encephalopathy consisted of stupor, headache and increased myoclonus. When the vitamins were restarted, clinical symptoms resolved and sural nerve potential amplitude doubled (Penn et al. 1992). This study did not

identify either vitamin as solely responsible and the mechanism for the clinical response is not clear.

Dosages of riboflavin for treatment of OXPHOS disorders have ranged from 9 to 300 mg/day. Since there have not been adverse reactions associated with riboflavin administration, no tolerable upper intake levels have been established (National Academy Press 2000). The results of treatment are varied, but demonstrate that in some patients with complex I deficiency, supplemental riboflavin alone or in combination with other supplements may provide some benefit.

THIAMINE

Thiamine functions as a coenzyme necessary for the oxidative decarboxylation of both pyruvate and α-ketoglutarate. The use of thiamine in the treatment of some forms of pyruvate dehydrogenase (PDH) deficiency has been well established, but thiamine effectiveness in the treatment of OXPHOS disorders is unclear. The therapy is postulated to improve aerobic glycolysis by enhancing pyruvate decarboxylation. An improvement in plasma lactate and pyruvate levels in patients with Kearns-Sayre syndrome was noted when thiamine was administered in doses of 300 mg/day (Lou 1981). It has also been reported that some patients with lactic acidemia improve clinically after high doses of thiamine (Duran and Wadman 1985). Most patients that respond have been found to have defects in the pyruvate dehydrogenase complex (PDHC) and, in particular, $E_1\alpha$ subunit mutations which contain the thiamine binding site

(Naito et al. 1994). It is not known whether this amelioration is disease-specific, or if it may have implications for other cases of lactic acidosis not caused by a PDHC defect.

Familial thiamine deficiency has been reported in two siblings with the 3243 MELAS mtDNA mutation; both presented with a skeletal muscle myopathy. Thiamine therapy (75 mg/day) improved the myopathy, normalized creatine kinase, and decreased blood lactate and pyruvate levels. Pyruvate dehydrogenase complex activity was below normal in both patients before thiamine treatment, but it is unclear whether the decreased PDH activity was primary or secondary, caused by the thiamine deficiency. This is the first description of a 3243 mtDNA mutation associated with thiamine deficiency (Sato et al. 2000). The authors question whether thiamine metabolism is altered, and suggest that thiamine status be investigated in patients with the 3243 mtDNA mutation.

In OXPHOS defects, thiamine has generally been used in combination with other cofactors with variable results. Dosages have ranged from 25 to 300 mg/day and there are no reported side effects with administration.

NIACIN

Nicotinamide, the amide form of niacin or nicotinic acid, is a precursor for both nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Although NAD and NADP undergo reversible reduction to NADH and NADPH, their functions in the cell are

quite different. The major role of NADH is to transfer electrons from metabolite intermediates to the respiratory chain. Complex I accepts electrons from NADH and passes them to ubiquinone. The rationale for nicotinamide use in OXPHOS disorder is to increase the cellular NADH and NAD concentration, and thereby enhance the substrate availability to complex I. In a case report of a MELAS patient with decreased complex I activity, nicotinamide treatment (1 g qid) resulted in marked decreases (50%) in both serum lactate and pyruvate. Blood NAD levels increased 24-fold during six weeks of treatment. The cellular NAD increase occurred in a time and dose-dependent manner in both control and patient fibroblasts, indicating the increase was probably universal. The authors speculate that the complex I defect led to an altered interaction between complex I and NADH, and although the affinity of complex I for NADH (determined by analyzing the complete progress curve of the reaction) was similar to that seen in control subjects, the nicotinamide supplementation enhanced the complex I activity by providing an excess of NADH. It is discouraging to note that the clinical effect was temporary and the patient eventually died (Majamaa et al. 1996).

Nicotinamide has been used in the treatment of OXPHOS disorders in combination with other cofactors. In a patient with the 3243 MELAS mtDNA mutation, treated with both riboflavin and nicotinamide, clinical improvement was noted, but the vitamins were not tested individually (Penn et al. 1992). Striatal lesions induced by malonate, a complex II

inhibitor, were blocked by the administration of CoQ_{10} and nicotinamide in an animal model. A combination of the two was more effective than either compound administered separately, as shown by lesion size and magnetic resonance imaging (Beal et al. 1994). CoQ_{10} and nicotinamide administration in large doses has been shown to reverse bioenergy changes in aged Drosophila; nicotinamide was more effective in reducing short-term mortality and increasing life span than CoQ_{10} (Driver and Georgiou 2002).

The role of nicotinamide in the treatment of OXPHOS disorders is not clear. Adverse side affects of supplemental niacin use, such as flushing and nausea, are usually associated with doses of greater than 1500 mg/day, but nicotinamide does not exhibit toxic effects (National Academy Press 2000).

VITAMIN K (PHYLLOQUINONE AND MENADIONE)

Vitamin K has been utilized in the treatment of patients with OXPHOS defects because it is assumed to mediate electron transport from NADH to electron acceptors such as coenzyme Q or cytochrome *c*. Vitamin K_3 (menadione) has been administered in combination with vitamin C (ascorbate) to donate electrons directly to cytochrome *c*. Menadione (80 mg/day) plus ascorbate (4 g/day) improved cellular phosphate metabolism (as measured by ^{31}P-NMR) in a patient with complex III deficiency (Eleff et al. 1984). The pre-treatment rate of recovery from exercise was 2.5% of normal; after administration of menadione and ascorbate, recovery rate

increased 21-fold (Eleff et al. 1984). Clinical and metabolic improvement continued at one-year follow-up, but symptoms deteriorated upon withdrawal of treatment. Recovery of function occurred after reinstatement of the vitamins (Argov et al. 1986). In another case study, a 16-year-old girl with complex III deficiency was treated with 40 mg/day of menadione and 4 g/day of ascorbate; although there was a mild improvement of her ataxia, there was no change in lactic acidosis and only slight improvement of muscle bioenergetics. Brain ^{31}P-MRS indices returned to normal after five months of treatment (Toscano et al. 1995).

Vitamin K_3, in the presence of complex I inhibitors, has been shown to stimulate oxygen utilization in mitochondria, resulting in an increase in NADH oxidation (Cooper et al. 1992). Experiments have been done to demonstrate the effectiveness of menadione, and menadione plus ascorbate, in reactivating drug-inhibited respiration (utilizing antimycin-A) and increasing the rate of ATP synthesis. Mitochondria stimulated by menadione were only inhibited by 25% using antimycin-A, whereas the combined therapy produced a 60% inhibition (Warshaw et al. 1966). Adding 5 μmol/L of menadione to the incubation medium of fibroblasts (that were inhibited in complexes I and III by rotenone and antimycin) resulted in a normalization of lactate to pyruvate ratios — suggesting a restoration of NADH oxidation (Wijburg et al. 1991). High dose menadione treatment (10 mg/kg/day) in a infant deficient in complex I resulted in improvement in clinical symptoms and biochemical parameters; this lends

support to the use of menadione in this multisystem disorder with lactic acidosis (Wijburg et al. 1989).

It is not clear which form of vitamin K is preferred for treatment of OXPHOS diseases, as K_1 (phylloquinone) has not been utilized in any of the reported studies. Phylloquinone has been shown to have better tissue retention and to reach higher levels in the mitochondria. Menadione is water-soluble, and must be alkylated to menaquinone-4 to be biologically active; phylloquinone, in contrast, is lipid-soluble and biologically active (Suttie 1985). Menadione has been demonstrated to result in hemolytic anemia and hyperbilirubinemia in newborns, whereas no side effects have been reported with phylloquinone use (Shoffner 2001). It is not known whether K_1 (phylloquinone) will have any benefit in the treatment of OXPHOS disorders.

CARNITINE

Carnitine functions to transfer long chain fatty acids across the mitochondrial membrane. It also facilitates branched chain α-ketoacid oxidation, shuttles acyl CoA products of peroxisomal β-oxidation to the mitochondrial matrix of liver, increases CoA levels in the mitochondria, and esterifies potentially toxic acyl CoA metabolites which impair the citric acid cycle (De Vivo and Tein 1990). Carnitine may also play a role in membrane stabilization by altering the physiologic properties of mitochondrial membranes (Carter et al. 1995).

Plasma and tissues levels of carnitine are maintained by exogenous dietary sources and *de novo* synthesis. It is estimated that approximately 75% comes from the diet, with red meat and dairy products being the primary sources (De Vivo and Tein 1990). Endogenous carnitine is synthesized in the liver and kidney from the amino acids lysine and methionine. Skeletal muscle (which stores approximately 90% of body carnitine) and heart muscle (which contains the highest concentration per gram of tissue) are unable to synthesize carnitine and must rely on uptake of carnitine from the blood (Angelini et al. 1992).

Decreased skeletal muscle and plasma carnitine levels have been reported in many cases of mitochondrial myopathies (Ogasahara et al. 1985; Hsu et al. 1995; Ogle et al. 1997), although this deficiency is assumed to be secondary to the mitochondrial defect. The carnitine deficiency may be apparent only in muscle, with the level of carnitine being normal in plasma. In cytochrome *c* oxidase deficiency, maximal rates of carnitine uptake were decreased from 20% to 47% of normal when studied in cultured skin fibroblasts (Tein et al. 1993). The authors postulate that the reduction in intracellular ATP may interfere with the functioning of the carnitine transporter, resulting in decreased intracellular carnitine levels (Tein et al. 1993). In one study, abnormal carnitine distribution in muscle was found in 29% (22 of 77) of patients with mitochondrial myopathy (Campos et al. 1993). Total and free carnitine levels were decreased while the level of long chain acyl carnitine was

elevated, possibly due to impaired transport of long chain fatty acids into the mitochondria, or to abnormal oxidation, or both (Campos et al. 1993).

Lipid storage myopathy is often attributed to carnitine deficiency, which impairs transport of fatty acids across the mitochondrial membrane. Only 31.5% of patients with lipid storage myopathy had muscle carnitine deficiency, indicating β-oxidation defects or mechanisms other than carnitine deficiency as a cause of fatty acid accumulation (Campos et al. 1993). Conversely, 25.6% of patients with muscle carnitine deficiency did not demonstrate abnormal lipid storage. The same pattern of abnormal carnitine distribution (increased acyl carnitine and decreased free carnitine) in muscle has been reported in 11 of 13 patients with idiopathic inflammatory myopathy (Arenas et al. 1996). Six of the 11 patients demonstrated histochemical or biochemical signs of mitochondrial dysfunction. A mechanism proposed is that impaired mitochondrial function could produce acyl CoA accumulation and increase carnitine esterification, resulting in a low free carnitine level (Arenas et al. 1996). Elevated levels of acyl CoA intermediates have also been suggested as impairing the function of adenine nucleotide translocase, which exchanges ADP for ATP across the inner mitochondrial membrane. Studies of mitochondrial myopathies have revealed similar carnitine distribution in plasma. Carnitine insufficiency (elevated ratio of esterified to free carnitine) has been found in the plasma of 43.8% (21 of 48) of patients with mitochondrial myopathies; both free and total carnitine deficiencies

were detected in 8.3% of the patients (Campos et al. 1993). Carnitine supplementation at 50 to 200 mg/kg/day was instituted; subjective improvement in muscle strength and tone were noted in 95% (20 of 21) of the patients (Campos et al. 1993). Echocardiographic and clinical evaluation improved in all eight patients with cardiomyopathy (Campos et al. 1993).

Memory loss with aging in animal and human studies has been associated with oxidative damage to lipids, proteins, and nucleic acids, and also with mitochondrial decay, which can disrupt neuronal function. The effects of being fed acetyl-L-carnitine and lipoic acid on cognitive function, brain mitochondrial structure, and oxidative damage were assessed in old rats. Both acetyl-L-carnitine and lipoic acid administration significantly improved performance on memory tasks, reduced brain mitochondrial structure decay, and reduced oxidative damage in the brain. The combination of the two metabolites produced the greatest improvement (Liu et al. 2002). It is postulated that both carnitine and lipoic acid may prevent mitochondrial decay in neurons associated with aging and help restore cognitive function.

Further research is warranted to establish the relationship of carnitine metabolism and mitochondrial function. Oral doses ranging from 100 mg/day up to 200 mg/kg/day of carnitine have been used in these diverse mitochondrial conditions. Side effects from large oral doses are minor, but include diarrhea and a fishy body odor in some cases. Carnitine

supplementation is generally recommended in conditions in which carnitine deficiency is suspected or identified, but the beneficial effect remain uncertain (Stanley 1987).

COMBINED THERAPY TREATMENTS

In a study of 16 patients with diverse mitochondrial disorders, a combination of vitamin K_3 (20–60 mg/day), ascorbic acid (1 g/twice a day), coenzyme Q_{10} (30–120 mg/day) and methylprednisolone (2–16 mg/every other day) was evaluated by ^{31}P-NMR, clinical and laboratory assessment (Peterson 1995). Length of follow-up varied from six months (death due to cardiac failure) to 13 years. A subset of the patients appeared to survive longer with fewer medical complications and functional disabilities than typically seen in clinical practice. Although the results are encouraging, the effectiveness of treatment is inconclusive.

In an open study in which 16 patients were on a treatment of coenzyme Q_{10} (300 mg/day), vitamin K_3 (60 mg/day), vitamin C (2 g/day), thiamine (100 mg/day), riboflavin (25 mg/day) and niacin (200 mg/day) for two months, and off treatment for two months, there was no significant, reproducible, objective clinical improvement (Matthews et al.1993). Serum lactate, exercise testing on a cycle ergometer, ^{31}PMRS studies at rest and after exercise and clinical follow-up were used as independent measures of oxidative metabolism. It is difficult to interpret the negative results of the study, but results from previous studies suggest that the two-month treatment period may have been too short for beneficial effects to become

evident. The results are further compromised by the small sample size — clinical observation and aerobic exercise performance were only assessed in ten patients. Further studies are required to evaluate the effectiveness of combined therapy in the treatment of mitochondrial disorders over a longer time period.

SUMMARY

Large double-blind placebo controlled trials of potentially beneficial therapies have been impossible to perform for a variety of reasons. Because of the rarity of the disorders, it is difficult to obtain sufficient numbers of patients to give statistical significance. The heterogeneity of both the phenotype and genotype, as well as the unpredictable natural history of the disease, has further complicated the issue. Since some beneficial effects have been demonstrated by cofactor therapy, and there is no proven effective treatment, it would be unethical to substitute a placebo. It is common practice to supplement with cofactor therapy, because there appears to be minimal or no side effects and potential exists for possible benefit. Based on the available information, it is difficult to draw definite conclusions regarding metabolic therapy. Most of the information is based on case studies, and the lack of reliable objective measurements has made the interpretation of results difficult. A review of the studies utilizing cofactor treatment is presented in Tables 2.1 and 2.2.

Serum lactate and pyruvate levels are often used as metabolic parameters to reflect oxidative metabolism, but they are frequently not

altered in OXPHOS disorders and may not demonstrate the therapeutic benefit of treatment. Mitochondrial dysfunction is most readily detected in muscle, but repeat muscle biopsies are an invasive and impractical method to monitor effects of therapy.

The need for further work is nowhere more evident than in the area of therapy. It is important to include measurements of clinical improvement in an effort to provide correlation between functional response and biochemical improvement. The difficulty arises as remissions and exacerbations are characteristic of OXPHOS disorders and may mask the efficacy of a particular treatment. This necessitates long-term studies to evaluate clinical effectiveness.

The ultimate goal of metabolic therapy is to increase ATP production, but it is not known if this goal is achieved through cofactor administration. The mechanism of action, the effect of individual and combined therapy, and the level of dosage of cofactor treatment remains to be determined. In combined cofactor treatment, it is difficult to determine the effects of individual therapy. The dosage and the site of the enzyme defect may affect which cofactors prove to be beneficial. In order to further elucidate the mechanisms of action of cofactor treatment, individual effects of each cofactor need to be evaluated by incubating control cells with the potential therapy and monitoring ATP production. This method could be used *in vitro* to study the effect of potential therapies and could be employed to design the optimal therapy for each individual

patient. Given the inherent problems mentioned above, and the difficulty of performing large clinical trials, individual trials where the patient serves as his or her own control may be a reasonable approach.

Table 2.1 Coenzyme Q_{10} Treatment in OXPHOS Disorders

Dose	Disorder	Study Details	Effects (Reference)
120 to 300 mg/day	MELAS	Case Report 1 patient 2 months	↓ CSF lactate and pyruvate Improved muscle weakness (Abe et al 1991)
150 mg/day	COX deficiency	Case Report 1 patient 24 months	↓ Serum lactate and pyruvate ↑ Post-exercise recovery (NMR) (Nishikawa et al 1989)
300 mg/day	MELAS	Case Report 1 patient 8 months	↓ Serum lactate and pyruvate Clinical improvement (Goda et al 1987)
60 to 120 mg/day	Kearns-Sayre syndrome	Case Report 1 patient 3 months	↓ Serum lactate and pyruvate Improvement in atrioventricular block and ocular movements (Ogasahara et al 1985)
90 mg/day	MELAS	Case Report 1 patient 14 months	↓ Serum lactate Improved muscle weakness (Yamamoto et al 1987)
210 mg/day	MELAS	Case Report 2 patients 8 months	Improved peripheral nerve function Improved muscle weakness (Ihara et al 1989)
200 mg/day	MELAS	Case Report 2 patients 2 weeks	Improved oxygen utilization during exercise (tissue oximetry) (Abe et al 1999)
150 mg/day	MELAS	Case Report 2 patients 10 months	↑ Post-exercise recovery (NMR) (Bendahan et al 1992)
150 mg/day	Mitochondrial myopathy	Open Study 9 patients 6 months	↓ Serum lactate and pyruvate (4 patients) (Chan et al 1998)
30 to 210 mg/day	MELAS	Open Study 11 patients 3-5 months	Improvement in neuromuscular symptoms (Andersen et al 1997)
150 mg/day	Mitochondrial myopathy	Open Study 8 patients 6 months	↑ Post-exercise recovery (NMR) (Effect mainly due to a single individual) (Gold et al 1996)

Table 2.1 Coenzyme Q_{10} Treatment in OXPHOS Disorders (cont)

Dose	Disorder	Study Details	Effects (Reference)
120 mg/day	Kearns-Sayre syndrome	Open Study 7 patients 12 months	↓ Serum lactate and pyruvate (Bresolin et al 1988)
160 mg/day	MELAS (4) MERFF (3) CPEO (1) (# of patients)	Double-Blind Crossover 8 patients 3 months	Improved global muscle strength (Chen et al 1997)
100 mg/day	Muscular Dystropy[1] Neurogenic Atropy[2]	Double-Blind Trials [1] 12 patients [2] 15 patients 3 months	Improvement in cardiac function and physical performance (Folkers et al 1995)
2 mg/kg/day	Mitochondrial myopathy	Double-Blind 44 patients 6 months	Reduction in post-exercise lactate levels (16 patients) (Bresolin et al 1990)
150 mg/day	MELAS (28 MIDD) (28 IGT) (15 NGT)	Open Study 44 patients 3 years	↑ Insulin secretory response and improved lactate response post-exercise (MIDD group)
200 to 3000 mg/day	Muscle CoQ_{10} deficiency	Case Report 6 patients 1 month	Improvement in strength, seizure control, muscle weakness and ataxia (Musumeci et al 2001)

MELAS; mitochondrial encephalopathy, lactic acidosis and stroke-like episodes
MERFF; myoclonic epilepsy, ragged-red fibers
CPEO; chronic progressive external ophthalmoplegia
MIDD; maternally inherited diabetes and deafness
IGT; impaired glucose tolerance
NGT; normal glucose tolerance

Table 2.2 Vitamin and Cofactor Treatment in OXPHOS Disorders

Cofactor	Dose	Disorder	Study Details	Effects (Reference)
Riboflavin	100 mg/day	Complex I deficiency	Case Report 1 patient 6 months	Improvement in exercise capacity (Arts et al 1983)
	50 mg/day	Complex I deficiency	Case Report 1 patient 3 years	Improved exercise tolerance and muscle tone (Ogle et al 1997)
	120 mg/day	Complex I deficiency	Case Report 1 patient 3 -17 months	↓ Serum lactate and pyruvate Improvement in motor development (Griebel et al 1990)
	9 to 60 mg/day	Complex I deficiency	Case Report 5 patients 6 months	Improvement in myopathic form (2/5) ↑ Complex I activity (Bernsen et al 1993)
Riboflavin plus Carnitine	9 mg/day 2 g/day	Complex I deficiency	Case Report 1 patient 7 months	Improved muscle weakness ↑ Complex I activity (Bernsen et al 1991)
Riboflavin plus Carnitine	200mg/day 2 g/day	Complex I deficiency	Case Reports 4 patients 1 – 2 years	Improvement in exercise capacity ↑ Complex I activity (1/4) (Scholte et al 1995)
Riboflavin Plus Nicotinamide	100mg/day 3 g day	MELAS	Case Report 1 patient 18 months	Improvement in encephalopathic symptoms and nerve conduction (NMR) (Penn et al 1992)
Vitamin K_3	10 mg/kg/ day	Complex I deficiency	Case Report 1 patient 3 months	↓ Serum lactate and pyruvate Clinical improvement (Wijburg et al 1989)

Table 2.2 Vitamin and Cofactor Treatment in OXPHOS Disorders (cont)

Vitamin K_3 plus Ascorbate	40 mg/day 4 g/day	Complex III deficiency	Case Report 1 patient 5 months	Improvement in ataxia (Toscano et al 1995)
Carnitine	50 to 200 mg/day	Mitochondrial myopathy (Carnitine deficiency)	Open Study 21 patients 1-24 months	Improvement in muscle strength Cardiac improvement (8/8 patients) (Campos et al 1993)
Combined Therapy	§	Diverse Mitochondrial Disorders	Case Reports 16 patients 1 to 13 years	Subset of patients appeared to have improved morbidity (Peterson et al 1995)
	ζ	Diverse Mitochondrial Disorders	Open Study 16 patients 8 months**	No significant reproducible objective improvement (Matthews et al 1993)

NAD; nicotinamide adenine dinucleotide
MELAS; mitochondrial encephalopathy, lactic acidosis and stroke-like episodes
CPEO; chronic progressive external ophthalmoplegia
§ CoQ_{10} (30 to 120 mg/day), vitamin K_3 (20 to 60 mg/day) ascorbate (2g/day) and methylprednisolone (2 to 16 mg every other day)
ζ CoQ_{10} (300 mg/day), vitamin K_3 (60 mg/day) ascorbate (2g/day), thiamine (100mg/day), riboflavin (25 mg/day), niacin (200 mg/day) 2 months treatment and 2 months off treatment

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CHAPTER 3

RESEARCH PLAN

RATIONALE

Dysfunction of oxidative phosphorylation (OXPHOS) has been implicated in a growing number of processes, including aging, and may underlie the most commonly encountered classes of degenerative diseases (Wallace 1992; Beal 1998). It has been estimated that about 1 in 5000 to 1 in 10,000 people will ultimately be diagnosed with some form of a mitochondrial disorder by the time they reach adulthood (Applegarth et al. 2000; van den Heuvel and Smeitink 2001). Marked progress has been made in the past 15 years in defining the specific biochemical defects and underlying molecular mechanisms of these defects, but limited information is available on the development and evaluation of effective treatment approaches.

Therapeutic trials in OXPHOS disorders are difficult to conduct because the diseases are rare and demonstrate vast clinical and genetic heterogeneity. Many reports of treatment have been anecdotal with relatively short follow-up. The unpredictable and variable natural history of these disorders and the lack of reliable clinical outcome measures, make it difficult to evaluate these reports. The goal of therapy is to increase mitochondrial ATP production and to slow or arrest the progression of clinical symptoms. The hope is that patients will survive longer with less functional disability and medical complications than typically seen in

clinical practice. Accumulation of toxic metabolites and reduction of respiratory activities have led to the use of antioxidants, electron transfer mediators (which by-pass the defective site) and enzyme cofactors in mitochondrial disease therapy. Metabolic therapies that have been reported to produce a positive effect include coenzyme Q_{10} (ubiquinone), other antioxidants such as ascorbic acid and vitamin E, vitamin K (phylloquinone and menadione), riboflavin, thiamine, niacin and carnitine. Arbitrary supplementation of single vitamins or cofactors may produce a relative deficiency in other cofactors; so combined cofactor therapy is carried out in an attempt to re-establish the proper balance of vitamins and cofactors required for metabolism to proceed normally.

Coenzyme Q_{10} has been reported to have a beneficial effect on clinical and biochemical outcomes in a variety of OXPHOS disorders. The benefits include: increased respiratory function; normalization of CSF and serum lactate and pyruvate; increased insulin response; improvement in atrioventricular block, ocular movements, EEG and neurological function; reduced muscle weakness; and decreased peripheral nerve damage (Ogasahara et al. 1985; Goda et al. 1987; Bresolin et al. 1988; Ihara et al. 1989; Abe et al. 1991; Suzuki et al. 1998). Most of these reports were case studies with limited numbers of patients, and with CoQ_{10} doses ranging from 30 to 300 mg per day.

Vitamin C and vitamin E have been utilized in OXPHOS disorders for their antioxidant properties (Przyrembel 1987). The α-tocopherol/

cholesterol ratio has been shown to be reduced in patients and in asymptomatic carriers of the 11778 Leber's hereditary optic neuropathy (LHON) mutation (Klivenyi et al. 2001). The authors conclude that the impaired function of complex I increases free radical formation and that the reduced ratio of α-tocopherol to cholesterol reflects α-tocopherol consumption in the affected tissues.

Riboflavin is the precursor for FMN and FAD, which are cofactors in complexes I and II. Supplementation of this cofactor has led to an improvement in exercise capacity and amelioration of clinical symptoms in patients with complex I deficiency (Arts et al. 1983; Bernsen et al. 1993; Scholte et al. 1995; Ogle et al. 1997).

Thiamine is a cofactor of pyruvate dehydrogenase and has been utilized to stimulate NADH production, which feeds into the respiratory chain at complex I. A patient with Kearns-Sayre syndrome demonstrated an improvement in plasma lactate and pyruvate levels upon thiamine supplementation (Lou 1981). However, in most OXPHOS defects, thiamine has been used in combination with other cofactors.

Nicotinamide is a precursor for both NAD and NADP. The major role of NADH is to transfer electrons from metabolite intermediates to the respiratory chain. Complex I accepts electrons from NADH and passes them to ubiquinone. The rationale for nicotinamide use in OXPHOS disorder is to increase the cellular NADH and NAD concentration, and thereby enhance the substrate availability to complex I. In a case report of

a MELAS patient with decreased complex I activity, nicotinamide treatment resulted in marked decrease (50%) in both serum lactate and pyruvate. Blood NAD levels increased 24-fold by six weeks of treatment (Majamaa et al. 1996).

Vitamin K_3 (menadione) has been administered in combination with vitamin C (ascorbic acid), based on the assumption that this will allow electrons to be donated directly to cytochrome *c* (Warshaw et al. 1966). This treatment was reported to improve cellular phosphate metabolism (as measured by ^{31}P-NMR) in a patient with complex III deficiency (Eleff et al. 1984). It is not clear which form of vitamin K is best for treatment of OXPHOS diseases, but phylloquinone has been shown to have better tissue retention and to reach higher levels in the mitochondria, as well as showing no side effects (Thierry et al. 1970; Suttie 1985).

Carnitine functions to transfer long chain fatty acids across the mitochondrial membrane. It also facilitates branched chain α-ketoacid oxidation, shuttles acyl CoA products of peroxisomal β-oxidation to the mitochondrial matrix of liver, increases CoA levels in the mitochondria, and esterifies potentially toxic acyl CoA metabolites which impair the citric acid cycle (De Vivo and Tein 1990). Decreased skeletal muscle and plasma carnitine levels have been reported in many cases of mitochondrial myopathies (Ogasahara et al. 1985; Hsu et al. 1995; Ogle et al. 1997), although this deficiency is assumed to be secondary to the mitochondrial defect. A mechanism proposed is that impaired

mitochondrial function could produce acyl CoA accumulation and increase carnitine esterification, resulting in a low free carnitine level (Arenas et al. 1996). Elevated levels of acyl CoA intermediates have also been suggested as impairing the function of the adenine nucleotide translocase, which exchanges ADP for ATP across the inner mitochondrial membrane. Carnitine supplementation is generally recommended in conditions in which carnitine deficiency is suspected or identified, but the beneficial effect remains uncertain.

Large double-blind placebo trials of potentially beneficial therapies have been impossible to perform for a variety of reasons. It is difficult to obtain sufficient numbers of patients to give statistical significance to these types of studies due to the rarity of the disorders. The heterogeneity of both phenotype and genotype, as well as the unpredictable natural history of the disease, has further complicated the issue. Since some beneficial effects have been demonstrated by cofactor therapy, and there is no other proven effective treatment, it would be unethical to substitute a placebo. It is common practice to supplement OXPHOS disease patients with cofactor therapy, because there appear to be minimal or no side effects, and the potential exists for possible benefit. Based on the available information, it is difficult to draw definite conclusions regarding metabolic therapy. Most of the information is based on case studies and the lack of reliable objective measurements has made the interpretation of results difficult. Serum lactate and pyruvate levels are often used as metabolic

parameters to reflect oxidative metabolism, but these metabolites may not demonstrate a response to the administered therapy. It is not known whether cofactor treatment improves oxidative phosphorylation, and there is limited information on the effect of treatment at the biochemical level. Mitochondrial dysfunction is most readily detected in muscle, but repeat muscle biopsies are an invasive and impractical method of monitoring the effects of therapy. The ultimate goal of metabolic therapy is to increase ATP production, yet it is not known whether an increase in cellular ATP levels result from the administration of the cofactor treatment. The mechanism of action, the effect of individual and combined therapies, and level of dosage of cofactors in metabolic treatment remain to be determined. The need for further work is nowhere more evident than in the area of therapy.

GOAL

The goal of the work described in this thesis is to develop objective measurements of the effect of cofactor treatment in mitochondrial disorders and to test the hypothesis that there will be an increase in cellular ATP production after cofactor treatment. This information will provide critical insights into the contribution of individual cofactors in increasing ATP synthetic capacity and may lead to development of better cofactor mixes that could improve the current therapeutic regimen for patients suffering from mitochondrial disorders.

OBJECTIVES

In this thesis, data will be presented that demonstrates that the measurement of ATP synthesis in permeabilized lymphocytes is an effective tool for monitoring the response to treatment and for evaluating the progression of disease.

(1) An *in vitro* method using lymphocytes has been developed to measure mitochondrial ATP synthesis before and after cofactor treatment. Permeabilized cells are incubated with (a) no substrate, (b) pyruvate and malate, (c) glutamate and malate, (d) succinate and rotenone, and (e) ascorbate and tetramethylphenylene diamine (TMPD). These selected substrates assess the ability of the mitochondria to synthesize ATP through the entire respiratory chain and also impart information about the site that is affected (Figure 3.1).

(2) Cofactors have been incubated with control lymphocytes to assess the effects of *in vitro* cofactor supplementation on mitochondrial ATP synthetic capacity.

Subjects

Subjects in whom a known mitochondrial defect has been identified were recruited for study after obtaining informed consent (Appendix I). Confirmation of the diagnosis was performed (using mitochondrial DNA studies and/or respiratory chain enzyme analysis) by independent accredited laboratories. (DNA analysis – Molecular Diagnostic Laboratory, Alberta Children's Hospital; DNA Diagnostic Laboratory, Montreal

Neurological Hospital; Emory Genetics Laboratory, Emory University School of Medicine) (Respiratory Chain Enzyme Analysis – Biochemical Diseases Laboratory, British Columbia Children's Hospital).

Treatment

After baseline measurements were obtained (clinical examination; biochemical parameters including plasma lactate, creatine kinase, amino acids, white blood cell count and differential, liver function tests, plasma carnitine and plasma CoQ_{10} levels; and lymphocytes collected for measurement of ATP synthesis), the subjects were supplied with the cofactor treatment. The daily cofactor treatment contained the following: coenzyme Q_{10} (5 mg/kg body weight); vitamin E (50 IU per 100 mg CoQ_{10}); vitamin K_1 (phylloquinone) (0.4 mg/ kg body weight); vitamin B complex (thiamine 25 mg, riboflavin 25 mg, niacinamide 25 mg, pyridoxine 25 mg, pantothenic acid 25 mg, biotin 25 µg, cyanocobalamine 25 µg, folic acid 1 mg, vitamin C 1000 mg); and carnitine (500 mg/day). Previous experience with this supplementation regime has provided practical methods for administering the cofactors; to date, no side effects have been reported. Compliance was monitored by calculation of daily dosages, prescription refills and measurement of plasma CoQ_{10}. Clinical evaluation, repeat blood work assessing biochemical parameters and CoQ_{10} levels, and measurement of ATP synthesis were performed at baseline, three, six and twelve months.

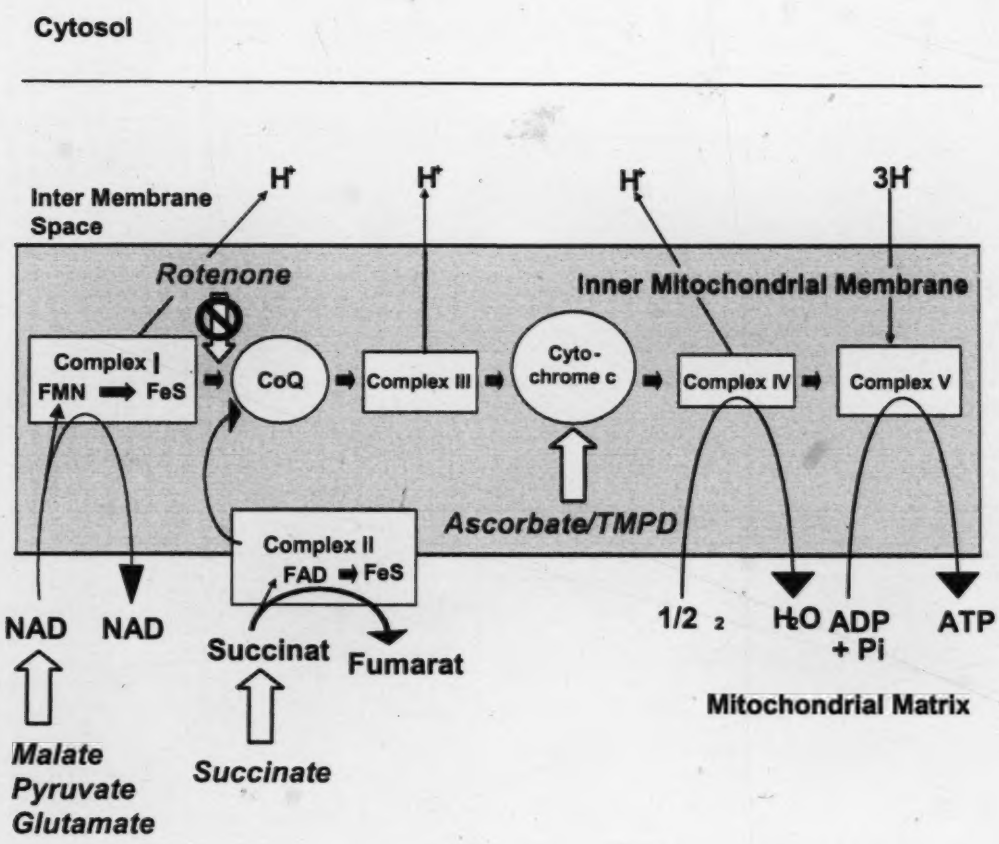

Figure 3.1 Measurement of ATP Synthesis in the Respiratory Chain

The amount of ATP produced with no added substrates is the result of oxidation of endogenous substrates. Pyruvate, malate and glutamate are NAD-linked substrates, allowing assessment of ATP synthesis through the entire respiratory chain. Rotenone, which blocks complex I activity, with addition of succinate can assess ATP synthesis through complexes II to V. Ascorbate and TMPD reduce cytochrome *c* directly, and thus measure the flux through complex IV.

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CHAPTER 4

THE USE OF LYMPHOCYTES TO SCREEN FOR OXPHOS DISORDERS

INTRODUCTION

Oxidative phosphorylation (OXPHOS) is responsible for producing most of the adenosine 5'-triphosphate (ATP) that is required by eukaryotic cells. Defects in OXPHOS encompass a large array of mitochondrial disorders, with onset of clinical symptoms occurring at any age (Munnich and Rustin 2001). Clinical recognition is initially difficult because defects in oxidative phosphorylation may present as encephalopathies, myopathies, neuropathies, or cardiomyopathies (Munnich et al. 1996). The clinical, biochemical and genetic heterogeneity has made the diagnosis and management of patients with OXPHOS defects exceedingly problematic.

More than 100 mutations in mtDNA have been identified over the past 13 years (Mitomap:http://www.gen.emory.edu/mitomap.html), most of them in association with OXPHOS disorders. Sequencing of the human mitochondrial genome, the use of Southern blot analysis and polymerase chain reaction (PCR) amplification have made possible the identification of some mitochondrial (mt) DNA defects in blood with no further diagnostic testing required. However, since the distribution of mutated mtDNA varies widely among tissues, it is often necessary to survey multiple tissues before finding a mutation in the mtDNA. In OXPHOS disorders caused by mtDNA deletions, the mtDNA mutation is often lost from blood due to replicative segregation, requiring a muscle biopsy for detection of the

deletion (Munnich et al. 1996). OXPHOS disorders may also be caused by mutations in the nuclear DNA or can occur as sporadic cases (Shoubridge 2001b). In the majority of cases, the molecular genetic defect remains unresolved.

Metabolic evaluation is often the first line of approach in screening patients with suspected OXPHOS disorders. A frequent indication of respiratory chain dysfunction is an abnormal redox state. This is partially due to the functional impairment of the citric acid cycle, which generates an excess of nicotinamide adenine dinucleotide phosphate (NADH); this results in a relative decrease of nicotinamide dinucleotide (NAD) due to a defect in the respiratory chain (Horton et al. 1996). This NADH excess in turn causes a secondary elevation in lactate and an increased lactate/pyruvate ratio. In addition, pyruvate may be transferred to the cytosol as alanine, causing hyperalaninemia (Horton et al. 1996). Although an increased ratio of blood lactate to pyruvate, accompanied by an increased ratio of 3-hydroxybutyrate to acetoacetate (>2:1), is highly suggestive of an OXPHOS defect (Munnich et al. 1996; Robinson 2001), some investigators have not found it particularly helpful (Jackson et al. 1995).

OXPHOS enzyme analysis in patients with presumptive mitochondrial dysfunction provides a more general diagnostic approach when specific mutations are not identified. Presently however, there is no single laboratory test that is routinely available to screen patients for

OXPHOS defects. Muscle biopsies, skin fibroblasts, or lymphoblast cell lines have been the material of choice for biochemical studies, but none of these are accessible as routine procedures in a standard clinical laboratory. As conclusive diagnostic evidence of an OXPHOS disorder often requires a muscle biopsy for enzyme analysis, it would be beneficial to have a less invasive and more accessible screening tool.

Cultured lymphoblasts and cultured skin fibroblasts have been utilized in the diagnosis of OXPHOS defects by examining the redox state of the cell, the activities of the respiratory chain enzymes, and ATP synthesis in permeabilized cells and isolated mitochondria (Robinson 1996). Cultured skin fibroblasts are useful in the diagnosis of OXPHOS disorders, but are time-consuming and result in a substantial delay in diagnosis (Bourgeron et al. 1992). The alternative approach of using lymphocytes transformed by Epstein-Barr virus (EBV) is hampered alterations to aerobic metabolism that are induced by cell transformations.

Here, a screening method is presented that utilizes circulating lymphocytes to detect OXPHOS dysfunction by examining the ability of mitochondria to synthesize ATP when provided with selected substrates. This method is an adaptation of previously described assays with cultured skin fibroblasts (Wanders et al. 1993; Robinson 1996). The use of substrates that enter the respiratory chain at different levels also imparts information about the site that is affected. The method is an effective screening tool — it requires only a small amount of blood (< 5mL), can be

completed in a few hours, and allows for repeated measurements. Due to the minimal invasiveness of blood collection, this method could provide an objective tool for monitoring response to treatment and evaluating progression of the disease, in addition to becoming an effective first screen in cases of suspected mitochondrial dysfunction.

MATERIALS AND METHODS

Subjects

This study was approved by the Health Research Ethics Board, Department of Medicine, University of Alberta Hospital (Appendix I). Informed consent was obtained from both control subjects and patients. Lymphocytes were isolated from healthy control individuals and from patients with known mitochondrial disorders, confirmed by the presence of mtDNA mutations or by respiratory chain enzyme analysis. ATP synthesis was measured in ten control subjects. Normal control blood samples were obtained from healthy laboratory volunteers at the same time that blood was collected from patients and were included in each analysis. A minimum of two determinations was analyzed from each sample. Eight patient samples were evaluated, representing five distinct mitochondrial disorders: Leber's hereditary optic neuropathy (LHON) 11778; mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) 3243; chronic progressive external ophthalmoplegia (CPEO); neurogenic ataxia and retinitis pigmentosa (NARP); and cytochrome *c* oxidase (COX) deficiency (Table 4.1).

Table 4.1 Patient Clinical and Diagnostic Data

Patient	Sex	Age	Diagnosis	Clinical Symptoms
1	M	45	LHON 11778	Visual loss at age 34 MS-like symptoms at age 44
2	M	40	LHON 11778	Visual loss at age 30
3	F	27	MELAS 3243	Hearing loss Hypertension
4	F	61	MELAS 3243	Hearing loss Chorioretinal dystrophy Diabetes Stroke-like episodes Dementia
5	M	46	CPEO 7 kb deletion	Ptosis Ophthalmoplegia Myopathy Mild hearing loss
6	M	43	CPEO 5 kb deletion	Mild Ptosis Ophthalmoplegia Myopathy Complete hearing loss
7	F	7	NARP (T8993G)	Encephalomyopathy Ataxia Developmental Delay Retinitis pigmentosa
8	M	12	COX deficiency (muscle and fibroblasts)	Encephalomyopathy Ataxia Developmental Delay

ATP Synthesis

Heparinized blood (5 mL), mixed with phosphate-buffered saline (PBS), was layered over Ficoll-Paque™ and centrifuged at 1750xg for 20 minutes (Fotino et al. 1971). Following centrifugation, the lymphocyte band was removed and any remaining red blood cells and residual Ficoll-Paque™ were removed by lysis with deionized water. The lymphocyte pellet was re-suspended in medium containing 50% modified Eagle's medium (MEM), 40% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO), then stored at -80°C. ATP synthesis was initially assessed using fresh lymphocytes, because the effect of freezing on cell viability and ATP synthesis had not been evaluated. To test cell viability, a 50 µL cell suspension of lymphocytes in sucrose medium (0.25 M sucrose, 5 mM Tris-HCl, 2 mM EDTA, pH 7.4) was added to 50 µL PBS. To this mixture, 0.2% trypan blue was added and the mixture was allowed to stand for 5 minutes. Cells that are not viable stain blue; the percentage of viable cells was then measured quantitatively. Comparison of data from fresh and previously frozen lymphocytes indicates a slightly lower rate of ATP synthesis with frozen samples, which is accounted for by the lower cell viability (86% ± 2%) caused by the freezing procedure (data not shown).

For the data presented here, frozen lymphocytes were re-suspended in MEM and pelleted by centrifuging at 2000xg for 10 minutes. After removal of MEM, the cells were washed twice with sucrose medium and centrifuged at 2000xg for 10 minutes. Aliquots were removed for

protein determination (Lowry et al. 1951). The cells were re-suspended in a digitonin incubation buffer (150 mM KCl, 25 mM Tris-HCl, 2 mM EDTA, 10 mM KH_2PO_4, pH 7.4, with 0.1% BSA (w/v), 1 mM ADP and 40 μg/mL digitonin) and divided into five 500-μL aliquots. Each aliquot was incubated with: (1) no added substrate; (2) 1 mM pyruvate, 1 mM malate; (3) 5 mM glutamate, 1 mM malate; (4) 10 mM succinate, 25 μg/mL rotenone; and (5) 10 mM ascorbate, 0.1 mM tetramethylphenylenediamine (TMPD) (Robinson 1996). Cells were incubated at 37°C for 30 minutes and the reaction stopped by the addition of 17.5 μL of 1.6 M perchloric acid. Samples were centrifuged at 21,000xg for 5 minutes to remove cell debris and ATP synthesis in the supernatant fraction was determined fluorometrically with hexokinase and glucose-6-phosphate dehydrogenase, using a fluorescence spectrophotometer (Perkin-Elmer 650-10S) (Williamson et al. 1973). ATP synthesis in patients was expressed as a percentage decrease of the mean value of control activity.

RESULTS AND DISCUSSION

The adaptation of a standard ATP synthesis assay was utilized for circulating lymphocytes. Standard substrates that have been widely used for this type of assay were utilized: (1) no substrate, (2) pyruvate/malate, (3) glutamate/malate, (4) succinate/rotenone, and (5) ascorbate/TMPD. The amount of ATP produced with no added substrates is a result of the oxidation of endogenous substrates. Pyruvate, malate and glutamate are NAD-linked substrates that produce NADH, which is subsequently

oxidized by Complex I. NAD-linked substrates can assess ATP synthesis through the entire respiratory chain. Succinate, a flavin adenine dinucleotide (FAD)-linked substrate, with the addition of rotenone to inhibit complex I, can assess the efficacy of ATP synthesis through complexes II, III, IV and V (Trounce et al. 1996). The substrates TMPD and ascorbate reduce cytochrome *c* directly and thus measure the flux through Complex IV (Hofhaus et al. 1996). Decreased ATP synthesis with all respiratory substrates can be the result of a defect in cytochrome *c* oxidase, which is considered to be a rate-limiting respiratory chain enzyme, or a defect in ATP synthase (Robinson et al. 1986). Some investigators have suggested that a defect of complex I may be rate-limiting for the overall respiration process (Rustin et al. 1993). Investigations in fibroblasts demonstrate that complex I activity must be decreased by approximately 50% before it has a significant impact on ATP synthesis (Kirby et al. 1999). It is not known whether the same effect on ATP synthesis is present in lymphocytes. In brain synaptic mitochondria, a 25% inhibition of complex I activity was enough to compromise ATP synthesis (Davey et al. 1998). In contrast, a threshold of 72% was found for complex I in nonsynaptic mitochondria (Davey and Clark 1996). The ability of complex I to be rate-limiting appears to vary among different cell types and is dependent on cellular conditions.

The determination of the ATP synthetic capacity in lymphocytes from control subjects and patients with an identified mitochondrial defect

was performed using this method. A comparison of ATP synthesis in control subjects and patients is presented in Table 4.2.

Table 4.2 Comparison of ATP Synthesis in Control Subjects and Patients*

Subjects	No Substrate	Pyruvate/ Malate	Glutamate/ Malate	Succinate/ Rotenone	Ascorbate/ TMPD
Control[a]	**227 ± 12** *n=54*	**490 ± 21** *n=56*	**468 ± 20** *n=48*	**436 ± 15** *n=48*	**354 ± 19** *n=56*
1 LHON	**116 ± 6 (49)** *n=8*	**208 ± 7 (58)** *n=8*	**214 ± 8 (54)** *n=8*	**248 ± 5 (43)** *n=7*	**175 ± 5 (51)** *n=7*
2 LHON	**158 ± 6 (31)** *n=8*	**265 ± 13 (46)** *n=6*	**259 ± 17(45)** *n=6*	**255 ± 15 (42)** *n=6*	**241 ± 13 (32)** *n=6*
3 MELAS	**147 ± 6 (35)** *n=4*	**261 ± 12 (47)** *n=4*	**289 ± 5 (38)** *n=4*	**296 ± 6 (32)** *n=4*	**271 ± 4 (23)** *n=2*
4 MELAS	**160 ± 11 (30)** *n=2*	**246 ± 6 (50)** *n=2*	**283 ± 2 (40)** *n=2*	**273 ± 9 (37)** *n=2*	**264 ± 1 (25)** *n=2*
5 CPEO	**154 ± 11 (32)** *n=3*	**262 ± 3 (47)** *n=3*	**284 ± 15 (39)** *n=3*	**247 ± 15 (43)** *n=2*	**291 ± 20 (18)** *n=2*
6 CPEO	**131 ± 6 (42)** *n=2*	**263 ± 0 (46)** *n=2*	**326 ± 6 (30)** *n=2*	**225 ± 12 (48)** *n=2*	**210 ± 3 (41)** *n=2*
7 NARP	**141 ± 4 (39)** *n=2*	**260 ± 32 (47)** *n=2*	**323 ± 7 (31)** *n=2*	**233 ± 9 (47)** *n=2*	**229 ± 0 (35)** *n=2*
8 COX	**69 ± 5 (70)** *n=3*	**124 ± 4 (75)** *n=3*	**152 ± 4 (68)** *n=3*	**161 ± 6 (63)** *n=3*	**111 ± 6 (69)** *n=2*

( ) = % decrease from mean control value
n = number of determinations
a = 10 subjects
Values expressed as mean ± SEM
*nmol ATP synthesized/30min/mg protein
SEM calculated on number of subjects (10) for control lymphocytes, whereas SEM for the individual patients was based on the number of determinations.

All eight patients, each with a confirmed OXPHOS disorder, had significantly decreased ATP synthesis in lymphocytes with all substrates tested. To determine whether the assessment of ATP production in lymphocytes was specific for OXPHOS dysfunction, three patients with muscle weakness and non-specific myopathy were evaluated. In these patients, a mitochondrial defect could not be identified. The ATP synthetic capacity in the lymphocytes of these patients was within the range of normal control values (data not shown).

ATP Synthesis in Lymphocytes from LHON Patients

Leber's hereditary optic neuropathy typically manifests as a bilateral, acute-onset, central vision loss caused by a degeneration of the optic nerve and retinal ganglion cell layer (Wallace et al. 2001). In a small percentage of cases, patients may develop the clinical or neuroradiologic signs similar to those seen with multiple sclerosis (Nikoskelainen et al. 1995; Olsen et al. 1995). The LHON 11778 mutation causes an amino acid substitution in the ND4 subunit of complex I. Biochemical studies conducted on the defect associated with the 11778 mutation have yielded conflicting results. Complex I activity has been shown to be reduced from 0% to 50% relative to control values, but most reports do not show a statistically significant reduction (Larsson et al. 1991; Majander et al. 1991; Degli Esposti et al. 1994; Smith et al. 1994; Carelli et al. 1997). The 11778 mutation does alter mitochondrial respiration, as indicated by a 30% to 50% reduction in respiration with NAD-linked substrates (Larsson

et al. 1991; Majander et al. 1991; Majander et al. 1996). It has been postulated that the 11778 mutation alters interaction of complex I with ubiquinone. This causes a decrease in proton translocation and subsequent decrease in ATP synthesis. It also destabilizes ubisemiquinone intermediates, which in turn promote the production of damaging oxygen radicals (Degli Esposti et al. 1994). In the lymphocytes from the two subjects harboring the 11778 mutation, there was a 45% to 58% reduction in ATP synthesis using NAD-linked substrates. There was a 31% to 51%, under all other assay conditions, indicating an overall reduction in mitochondrial OXPHOS. Contrary to results obtained in the present study, other investigators using EBV-transformed lymphocytes from LHON patients have not found a decreased rate of oxidation when using succinate as a substrate (Larsson et al. 1991; Majander et al. 1991). It is not known whether this observed difference is due to the effect of cell transformation on mitochondrial activity, or to the influence of different nuclear backgrounds. Mitochondria with the same genotype have been shown to have varied rates of respiration in different nuclear backgrounds (King and Attardi 1989).

ATP Synthesis in Lymphocytes from MELAS Patients

Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) is a progressive neurodegenerative disease characterized by the symptoms described in the acronym. Additional features may include sensorineural deafness, pigmentary retinopathy, type II diabetes, seizures,

recurrent vomiting, dementia, hypertrophic cardiomyopathy, limb weakness and myopathy (Goto et al. 1991; Ciafaloni et al. 1992). It is estimated that 80% of MELAS patients have a heteroplasmic A to G point mutation in the dihydrouridine loop of the $tRNA^{leucine}$ gene at base pair (bp) 3243 (Kobayashi et al. 1990; Enter et al. 1991). The mtDNA encoded polypeptides with the highest proportion of leucine residues are in the complex I subunits, ND6 and ND3 (Goto et al. 1992). Biochemical studies indicate that complex I deficiency is the defect most commonly observed in MELAS (Goto et al. 1992; Morgan-Hughes et al. 1995). In a study examining the phenotype-genotype correlation of the 3243 MELAS mutation, cytochrome aa_3 content was decreased in six out of 14 patients; in one case, cytochrome *b* was also reduced (Morgan-Hughes et al. 1995). In the same study, four of the 14 patients showed a polarographic defect involving complexes I and III. Complex III and IV deficiencies alone, or in combination with a complex I deficiency, have also been observed in studies of MELAS patients (Koo et al. 1993). The two MELAS patients in this study have the most marked reduction (38% to 50%) in ATP synthetic capacity with NAD-linked substrates, which reflects an impaired complex I activity. A 32% to 37% reduction in ATP synthesis was also observed with succinate and rotenone, which measures ATP synthetic capacity through complexes II, III, IV, and V. The flux through complex IV, as measured by ascorbate and TMPD, was reduced by about 25%. Thus, impaired mitochondrial function in the lymphocytes from the two MELAS patients

was clearly demonstrated. As with the LHON 11778 mutation, the mtDNA defect in these patients was detected in blood.

ATP Synthesis in Lymphocytes from CPEO Patients

Chronic progressive external ophthalmoplegia encompasses a wide range of symptoms that include ophthalmoplegia, ptosis and myopathy (Wallace et al. 2001). The majority of cases are sporadic and the patients are heteroplasmic for the normal and rearranged mtDNA molecules. These rearrangements frequently present as a deletion between nucleotide 8469 and 13447, removing approximately 5 kilobases of mtDNA (Wallace et al. 2001). This region spans from the ATPase 8 gene to the ND5 gene and affects the function of complexes I, III, IV and V. High proportions of deleted mtDNA inhibit cell replication; as a consequence, most bone marrow cells affected by the deletion stop replicating, leaving only normal bone marrow cells to grow (Shoffner 2001). As a result, molecular diagnosis of the mtDNA deletion in virtually all CPEO patients requires analysis of a post-mitotic tissue such as muscle to establish a definite diagnosis. The analysis of ATP synthesis in isolated lymphocytes from the two CPEO patients in this study demonstrates an 18% to 48% reduction in ATP synthetic capacity. In both of the patients, however, the mtDNA defect was only detected in skeletal muscle. The author is aware of only one other report of mitochondrial dysfunction in CPEO patients detected in extramuscular tissue. In that study, a 30% to 50% decrease in maximal

respiration rate in mononuclear cells was detected, using a sensitive oxygraphic assay (Kunz et al. 1995).

Reports of presumably sporadic cases of CPEO, or of families in which maternal inheritance is ruled out, point to the involvement of nuclear genes affecting the mtDNA in these patients. Familial cases of CPEO have recently been shown to result from mutations in the adenine nucleotide translocator (ANT 1), Twinkle and polymerase gamma (POLG) genes (Hirano et al. 2001). Mutations in POLG, the DNA polymerase responsible for mtDNA replication and base excision repair, have been associated with both autosomal-dominant and autosomal-recessive variants of CPEO, with the common 4977 bp deletion found in both forms of the disease (Ponamarev et al. 2002). Mutations in POLG affect the mtDNA replication machinery, resulting in defective oxidative phosphorylation and consequent decreased ATP synthesis (Van Goethem et al. 2001). Autosomal mitochondrial disorders exhibiting deletions and rearrangements have a higher mtDNA mutation rate due to enhanced damage, or to compromised mechanisms of mtDNA maintenance. In autosomal CPEO patients, the deletions are probably generated *de novo* as somatic mutations in post-mitotic tissues, due to mutations in nuclear genes encoding proteins required for mtDNA maintenance (Zeviani et al. 1989; Van Goethem et al. 2001). In both of the CPEO patients in this study, it is possible that the defect is inherited in an autosomal manner. Therefore, it is plausible that the deletions found in the muscle of these

patients reflect an inherited nuclear gene defect that affects mtDNA integrity in post-mitotic tissues. The mitochondrial function in extramuscular tissues would thus be compromised, but the deletion would be lost from these tissues. This could explain the decreased ATP synthesis found in the lymphocytes of the CPEO patients, even though the deletion could only be detected in muscle.

ATP Synthesis in Lymphocytes from a Patient with Neurogenic Ataxia and Retinitis Pigmentosa

The NARP mutation may present with neuropathy, ataxia and retinitis pigmentosa, and is also a frequent cause of Leigh syndrome (sub-acute necrotizing encephalopathy) (Shoffner 2001). The T8993G NARP mutation changes the highly conserved leucine in the ATP6 gene to an arginine. Data from an *E.coli* model for this mutation indicate that the amino acid change would compromise the ATP synthase proton channel (Wang and Oster 1998). Biochemical analysis of the T8993G mutation in fibroblasts and lymphoblasts has shown a 30% to 50% reduction in ATP synthesis (Tatuch and Robinson 1993; Makela-Bengs et al. 1995). A similar reduction (31% to 47%) in ATP synthesis was observed in the lymphocytes from the NARP patient included in this study.

ATP Synthesis in Lymphocytes from a Patient with Cytochrome c Oxidase Deficiency

Complex IV (COX) is the terminal enzyme in the mitochondrial respiratory chain. It catalyzes the reduction of molecular oxygen and pumps protons across the inner membrane to contribute to the electrochemical gradient

used to synthesize ATP (Shoubridge 2001a). It is considered by some investigators to be the rate-limiting enzyme in the respiratory chain (Robinson et al. 1985). Patients with COX deficiency can present with a variety of clinical phenotypes including Leigh syndrome, cardiomyopathy and myopathy (Shoubridge 2001a). It has been demonstrated that the rate of ATP synthesis is decreased with all substrates in COX deficiency (Robinson 1996). In the lymphocytes from the COX-deficient patient, there was a 63% to 75% reduction in ATP synthesis. The COX deficiency in this patient has only been detected in muscle and skin fibroblasts to this point. Screening for common mutations in the mtDNA of both blood and muscle has not revealed any mutations, leading to the assumption that the enzyme deficiency is a result of an unidentified nuclear gene mutation.

Regardless of the mechanism of an OXPHOS disorder, the end result is a reduction in the production of ATP, resulting from impairment of the respiratory chain. Decreased ATP synthetic capacity in the lymphocytes of patients with five distinct mitochondrial disorders was demonstrated in this study. The measurement of ATP synthesis in permeabilized lymphocytes is thus likely to be an effective screening tool for detecting OXPHOS dysfunction. This is the first report of measurement of ATP synthetic capacity in circulating lymphocytes of mitochondrial patients. Until now, all published data regarding fluorometric determination

of ATP synthesis has only been measured in cultured cells or muscle mitochondria.

Lymphocytes are an ideal system for studying the function of the respiratory chain as 85% of the ATP produced is from oxidative phosphorylation and 15% from glycolysis (Roos and Loos 1973). Investigation of the mononuclear cell preparation indicated that approximately 90% of the cells were lymphocytes (data not shown). The ATP synthesis value was therefore based on total cellular protein, instead of number of mononuclear cells, since total protein includes the contribution of monocytes and granulocytes in the cell preparation. Traditionally, plasma lactate measurements have been utilized as a screening tool. Pitfalls in the metabolic screening approach are numerous. They include: (1) an artificial elevation of lactic acid due to improper collection and processing of the blood sample; (2) proximal renal tubulopathy leading to lower blood lactate and increased urinary lactate; (3) an elevated lactate may be revealed only by glucose loading and exercise testing; and (4) the defect may be tissue-specific, barely altering the redox state in plasma. These metabolic investigations are further complicated by the fact that a number of OXPHOS defects present with normal blood lactate levels. In an investigation of 51 patients with respiratory chain disorders, only 40% had elevated fasting blood lactate levels (Jackson et al. 1995). The best-known examples of normal plasma lactate in mitochondrial diseases are LHON and CPEO, while NARP can

also present with normal lactic acid levels in the later onset group and in patients with milder forms of the disease (Robinson 2001). Normal blood values of plasma lactate, pyruvate, and alanine, concomitant with elevations of these metabolites in cerebral spinal fluid (CSF), have also been observed in patients with MELAS (Abe et al. 1991).

Circulating lymphocytes have been used to study respiratory chain activity by examining cell respiration with polarography and with spectrophotometric measurements of OXPHOS complexes (Rustin et al. 1994). The limitations of polarographic studies are that the technique must be performed on fresh tissue (Munnich et al. 1996), and that conventional oxygen electrodes require large amounts of mitochondrial protein (1 to 10 mg) to obtain reliable results (Taylor and Turnbull 1997). Spectrophotometric studies on lymphocytes can assess complexes II + III, and complex IV activity, but are not reliable for the assessment of complex I activity (Rustin et al. 1994). The oxidation of NAD-linked substrates by detergent-treated or freeze-thawed cells is questionable due to the rotenone-resistant cellular NADH cytochrome *c* reductase activity (Munnich et al. 1996); a partial defect in complex I activity could thus remain undetected. The ability to detect complex I deficiency is important, as complex I deficiency may be the most common OXPHOS disorder encountered (Robinson 1993; Kirby et al. 1999). In addition, mtDNA mutations (such as MELAS, LHON and CPEO) are associated with complex I deficiency (Kirby et al. 1999). The inability to diagnose complex I

deficiency in lymphocytes is a limitation to using this easily accessible tissue for spectrophotometric studies. Generally, spectrophotometric investigations have utilized the muscle biopsy as the tissue of choice to further characterize respiratory chain activity.

Cultured skin fibroblasts and transformed lymphocytes have also been used to assess ATP synthesis. Cultured fibroblasts are useful in the assessment of OXPHOS disorders but result in a substantial time delay in the diagnosis, limiting their use as an effective screening tool (Bourgeron et al. 1992). In addition, the reliance of fibroblasts on glycolysis does not make them an ideal system to study oxidative changes. The alternative approach of using EBV-transformed lymphocytes is hampered by the alterations to aerobic metabolism that are induced by cell transformation. In EBV-transformed lymphocytes from a patient with Pearson syndrome, recovery of respiratory chain activity was noted, but the amount of deleted mtDNA (60%) did not change. Initially, the cells showed a marked reduction in cytochrome *c* oxidase and succinate cytochrome *c* reductase activity; three weeks after infection with EBV, succinate cytochrome *c* reductase activity was slightly higher than in control cells; after 80 days, cytochrome *c* oxidase was normal. The recovery of respiratory chain activity was attributable to an increase in the mtRNA translational efficiency (Bourgeron et al. 1993). Changes in mitochondrial activity observed upon cell transformation may be contributed to by: induction of nuclear and mitochondrial OXPHOS genes (Torroni et al. 1990); changes

in the organization of the mitochondrial genome (Kinchington and Griffin 1987); induction of Bcl-2, a mitochondrial protein associated with apoptosis (Henderson et al. 1991); and changes in the balance between metabolic pathways contributing to ATP synthesis (Tyller 1992). These alterations, however, suggest this is not an ideal means for assessing mitochondrial dysfunction.

In mtDNA mutation disorders, muscle and brain tissue are thought to contain higher percentages of heteroplasmy than fibroblasts and blood. In spite of this, OXPHOS dysfunction was detected in the blood of the patients with MELAS and NARP in this study. The ability to demonstrate decreased ATP synthesis in the lymphocytes of CPEO patients, where the deletion could only be detected in muscle, clearly expands the applicability of this screening method. Generally, OXPHOS dysfunction presents in highly oxidative tissues such as muscle, heart, eye and the central nervous system. Although the bioenergetic consequences do not frequently result in hematopoietic presentations, the findings in this study illustrate that a defect in ATP synthesis is present in circulating lymphocytes. The significance of decreased ATP synthetic capacity in lymphocytes in the absence of a hematopoietic phenotype in patients is not clear and requires further investigation.

The clinical spectrum of OXPHOS defects is large and extremely diverse. In a minority of cases, there are "classical" mitochondrial disorders such as LHON with a relatively straightforward clinical diagnosis

and a readily identifiable molecular defect. The majority of patients, however, have clinical features which are suggestive of mitochondrial disease but do not fit into a specific category (Chinnery et al. 1999). The most difficult group of patients, which is expanding in number, present with unexplained signs and symptoms; these patients are eventually diagnosed with a mitochondrial disorder (Chinnery et al. 1999). An increasing number of nuclear genes are being implicated in respiratory chain disorders and are the most frequent cause of OXPHOS disorders (Shoffner 2001). Although some candidate genes have been identified, the large numbers of unidentified mutations necessitates biochemical analysis for the diagnosis of these conditions.

A rapid screening assay for the detection of mitochondrial disorders using < 5ml of blood is presented. This method allows measurement of ATP synthesis — one of the most relevant parameters of mitochondrial metabolism. The use of substrates that enter the respiratory chain at different levels also provides an indirect measurement of the function of the various complexes. The only limitation is that this method does not characterize the specific enzyme defect. Given that a mitochondrial disorder is not obvious in an increasing number of patients, it is important to ascertain OXPHOS dysfunction by a relatively non-invasive procedure. This assay could complement clinical diagnosis and guide clinicians to more specialized, invasive diagnostic procedures. Lymphocytes are easy to obtain and the measure of ATP synthesis can serve as an effective

screening tool for the diagnosis of an OXPHOS disorder. These findings also provide a means of objectively evaluating possible therapies and monitoring progression of the disorder.

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CHAPTER 5

EFFECT OF COFACTOR TREATMENT ON ATP SYNTHETIC CAPACITY IN PATIENTS WITH OXPHOS DISORDERS

INTRODUCTION

Oxidative phosphorylation (OXPHOS) disorders, which are growing in number, are characterized by a decline in the ability to supply cellular energy requirements and by vast clinical and genetic heterogeneity. OXPHOS is unique because it is the only metabolic pathway controlled by both the nuclear and mitochondrial genomes. Characteristics of the mitochondrial genome (such as replicative segregation, threshold expression, and tissue-specificity) contribute to the broad spectrum of clinical presentations associated with defects in OXPHOS. Clinical features may include a combination of neuromuscular and non-neuromuscular symptoms with a progressive course and involvement of seemingly unrelated tissues. In fact, a respiratory chain deficiency can give rise to any symptom, in any organ or tissue, at any age, and with any mode of inheritance (Munnich and Rustin 2001).

Regardless of the specific defect, an OXPHOS disorder is marked by a decline in ATP synthetic capacity, a pertinent parameter of mitochondrial metabolism. The goal of nutritional cofactor therapy in OXPHOS disease is to increase mitochondrial ATP production and slow or arrest the progression of clinical symptoms. Accumulation of toxic metabolites and concomitant reduction of respiratory activities has lead to the use of antioxidants, electron transfer mediators and enzyme cofactors.

Coenzyme Q_{10}, other antioxidants such as ascorbic acid and vitamin E, riboflavin, thiamine, niacin, vitamin K (phylloquinone and menadione) and carnitine have been utilized to augment energy production in these disorders.

CoQ_{10} has been the most widely used supplement in the treatment of mitochondrial disorders. A number of reports describe improvements in physical performance (Folkers and Simonsen 1995), enhanced exercise tolerance (Goda et al. 1987; Bresolin et al. 1988; Nishikawa et al. 1989; Abe et al. 1999), decreased muscle weakness (Yamamoto et al. 1987; Ihara et al. 1989) and improvement in neurological function (Bresolin et al. 1988) with CoQ_{10} administration. In contrast, other studies have not demonstrated a significant improvement in biochemical parameters and clinical symptoms with CoQ_{10} treatment (Gold et al. 1996; Chen et al. 1997).

Treatments with riboflavin alone, or in combination with nicotinamide, have demonstrated an improvement in exercise capacity and resolution of encephalomyopathic symptoms in selected patients (Arts et al. 1983; Penn et al. 1992; Scholte et al. 1995). There are similar reports of clinical and metabolic improvement with high-dose vitamin K_3 and ascorbate administration (Eleff et al. 1984; Argov et al. 1986).

Most of the reports on the effect of cofactor treatment have been based on case studies with limited patient numbers and the lack of objective measurements has made the interpretation of results difficult.

There are limited numbers of controlled clinical trials with sufficient patients to evaluate effectiveness of therapy. Larger trials, examining the effect of combined cofactor treatment in a variety of mitochondrial disorders, were unable to confirm that the treatment significantly improved oxidative metabolism (Matthews et al. 1993; Peterson 1995). In most of the studies, the treatment period may have been too short for beneficial effects to become evident.

The present study was designed to test the hypothesis that there will be an increase in cellular ATP production after cofactor administration. The patients in this study were evaluated over a twelve-month period to assess the effectiveness of combined cofactor therapy over a longer time period than has been reported to date. It has been previously demonstrated that isolated lymphocytes are an effective tool for detecting and monitoring OXPHOS dysfunction (Chapter 4). In this study, twelve patients with known OXPHOS defects were evaluated and a significant improvement in ATP synthetic capacity in lymphocytes after cofactor treatment was demonstrated.

MATERIALS AND METHODS

Subjects

Patients with a clearly identified mitochondrial defect were recruited for the study after obtaining informed consent (Appendix I). The purpose and requirements of the study were fully explained to each subject before they gave consent to participate. The study was approved by the Health

Research Ethics Board, Department of Medicine, University of Alberta. Twelve patients were evaluated in this study (Table 5.1).

Table 5.1 Clinical and Diagnostic Data of Patients

Patient	Sex	Age	Diagnosis	Clinical Symptoms
RS	M	45	LHON 11778 Homoplasmic	Vision loss at age 33 Multiple sclerosis-like symptoms at age 44
DB	M	41	LHON 11778 Homoplasmic	Vision loss at age 30
CL	F	46	LHON 11778 Heteroplasmic	Visual problems at age 2
ML	M	15	LHON 11778 Heteroplasmic	Vision loss at age 14
TL	M	14	LHON 11778 Heteroplasmic	Visual problems at age 3
KM	M	9	LHON 11778 Heteroplasmic	Visual problems at age 5
LS	M	44	CPEO 5 kb deletion	Mild Ptosis Ophthalmoplegia Myopathy Complete hearing loss
PD	M	47	CPEO 7 kb deletion	Ptosis Ophthalmoplegia Myopathy Mild hearing loss
LO	F	36	CPEO 5 kb deletion	Ptosis Ophthalmoplegia Mild Myopathy
DH	F	26	MELAS 3243	Hearing loss Migraines Poor exercise tolerance
CP	F	58	NARP T8993G	Ataxia Retinitis Pigmentosa Peripheral Neuropathy Muscle Weakness Hearing Loss
SA	M	12	COX deficiency	Encephalomyopathy Ataxia Developmental Delay

These patients represent five distinct mitochondrial disorders: LHON 11778 (six patients), CPEO (three patients), MELAS 3243 (one patient), NARP (one patient), and an isolated COX deficiency (one patient). A detailed clinical history is provided in Appendix I.

Treatment

After baseline measurements were obtained (clinical examination, biochemical investigations, and lymphocytes collected for measurement of ATP synthesis), the subjects were supplied with cofactor treatment. The daily cofactor treatment contained the following: coenzyme Q_{10} (5 mg/kg body weight) and vitamin E (50 IU α-tocopherol per 100 mg CoQ_{10} softgel capsule) (Natural Factors®); vitamin K_1 (phylloquinone) (0.4 mg/kg body weight) (Sabex ®); vitamin B complex (thiamine 25 mg, riboflavin 25 mg, niacinamide 25 mg, pyridoxine 25 mg, pantothenic acid 25 mg, biotin 25 µg, cyanocobalamine 25 µg, folic acid 1 mg, vitamin C 1000 mg) (Natural Factors®); and carnitine 500 mg/day (Sigma-Tau®). The dosages administered were based on what was previously used in clinical practice and in consultation with Emory Genetics Clinic, Emory School of Medicine. Patients were instructed to consume well-balanced, nutritionally adequate meals and requested to discontinue any additional vitamin or mineral supplementation. Compliance was monitored by calculation of daily dosages, reviewing prescription refills and measurement of serum CoQ_{10}. No side effects from the therapy were reported and compliance was good. Clinical evaluation, repeat blood work assessing biochemical parameters

and CoQ_{10} levels, and measurement of ATP synthesis were performed at baseline, three, six and twelve months.

Biochemical Investigations

All blood samples were obtained two to four hours post-prandial from an antecubital vein. The Department of Laboratory Medicine, University of Alberta Hospital, performed laboratory analyses of plasma lactate, creatine kinase, amino acids, carnitine, white blood cell count and differential, as well as liver function tests. As peak plasma levels of CoQ_{10} are observed three to four hours after dosing (Wahlqvist et al. 1998), patients were requested to refrain from taking the CoQ_{10} supplementation on the day of the blood collection. Plasma CoQ_{10} was assessed by high performance liquid chromatography at Emory Genetics Laboratory, Emory University School of Medicine. Studies of ATP synthesis were performed as previously described (Chapter 4). Normal control blood samples were obtained from laboratory volunteers at the same time that blood was collected from patients and were included in each analysis. A minimum of two determinations was made for each sample.

Statistics

ATP synthesis in lymphocytes from patients was compared to that of control subjects. Comparisons between pre- and post-treatment ATP synthesis levels in patients were performed using the mean percentage change ± SEM and assessed using a two-tailed Student's t test. Significance was established at $p \leq 0.05$.

RESULTS

Clinical Evaluation

The clinical evaluation and management of patients with OXPHOS disorders is difficult due to the diverse nature of the disease and the unpredictable clinical course. Metabolic physicians in the Medical Genetics Clinic, University of Alberta, assessed the patients in this study, ranging in age from 9 to 58 years. The patients presented with a variety of clinical symptoms — ranging from isolated ocular disease to encephalopathy with neuromuscular involvement. The management of these patients (in addition to cofactor treatment) included medications to treat symptoms (seizures, neuropathic pain, cardiac dysfunction), prompt treatment of intercurrent viral illness, and monitoring of progress by physical and neurological examination. Clinical follow-up over the course of the study was performed at baseline, three, six and twelve months. Patients RS (LHON), LO (CPEO), and DH (MELAS) reported an increase in energy levels and improvement in exercise tolerance. Patients with decreased visual acuity, ptosis and ophthalmoplegia did not show any changes in their symptoms over the time period of the study, as assessed by a genetic ophthalmologist. Patient PD (CPEO) noted a gradual increase in muscle pain with exercise throughout the year-long study period. The remaining patients showed no other evidence of further deterioration or amelioration of clinical symptoms.

Biochemical Parameters

Laboratory analyses assessing metabolic status were performed at each time point. With the exception of patient DH (MELAS), all blood values were within the normal range. DH had a slightly elevated lactate of 2.6 mmol/L (Normal 0.5 to 2.2 mmol/L) at baseline; values were 2.4, 2.7, and 2.3 mmol/L at three, six and twelve months respectively. At the seven-month point during the study, patient PD experienced a marginally elevated lactate (2.4 mmol/L), and a marked increase in creatine kinase (1360 U/L; Normal $\leq$ 180 U/L) 24 hours post-exercise, but maintained levels within the normal range when tested at rest. Patient SA (COX deficiency) experienced transient elevated lactate and liver function enzymes levels with administration of valproate (Depekene®), a seizure medication; these levels normalized when the medication was discontinued.

Serum CoQ_{10} levels increased an average of 579% over the 12-month treatment period (pre-treatment levels: 0.62 $\pm$ 0.07 µg/mL; post-treatment levels: 4.21 $\pm$ 0.07 µg/mL, $p \leq 0.01$) (Figure 5.1; Table 5.2). The increase in serum CoQ_{10} confirmed good compliance with the prescribed therapy.

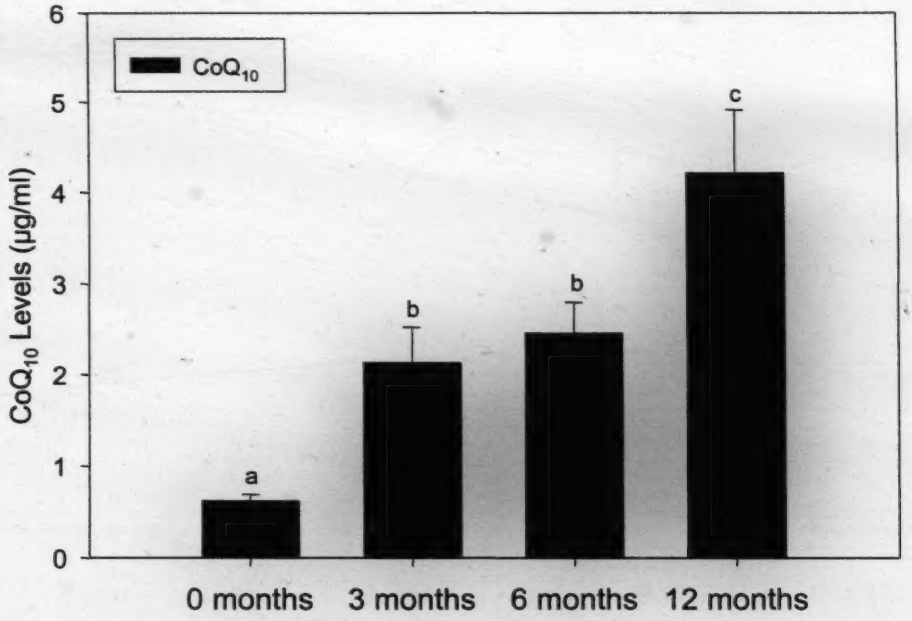

Figure 5.1 Plasma CoQ_{10} Levels (µg/ml)
Values are expressed as mean ± SEM. Values with different superscripts are significantly different ($p \leq 0.01$).

Table 5.2 Plasma CoQ_{10} Levels (µg/mL)

Month / Patients	0	3	6	12
RS	0.47	1.75	1.76	3.43
DB	1.10	1.97	3.41	5.47
CL	0.44	1.67	2.38	5.35
ML	0.68	1.75	4.45	7.39
TL	0.57	1.04	3.30	5.52
KM	0.54	2.02	2.21	1.35
PD	0.78	3.10	2.23	3.08
LS	0.78	1.30	1.39	2.47
LO	0.39	1.02	0.79	1.51
DH	0.52	4.72	3.30	5.54
CP	0.77	3.10	2.69	7.22
SA	0.42	NA	1.45	2.13
Average ± SEM	**0.62 ± 0.07**	**2.13 ± 0.39**	**2.45 ± 0.34**	**4.21 ± 0.70**

ATP Synthesis in Patient Lymphocytes

Oxidative phosphorylation is responsible for approximately 90% of the total ATP produced in eukaryotic cells containing mitochondria. Therefore, the measurement of ATP synthesis is pivotal for evaluating cellular energy metabolism in OXPHOS disorders. In the past, cultured fibroblasts and transformed lymphocytes have been used to assess mitochondrial ATP synthesis (Bourgeron et al. 1992; Robinson 1996). The use of cultured fibroblasts is time-consuming, and the reliance of fibroblasts on glycolysis does not make them an ideal system for studying oxidative changes. The alternative approach of using EBV-transformed lymphocytes is hampered by the alterations to aerobic metabolism that are induced by cell transformation (Bourgeron et al. 1993).

Circulating lymphocytes were utilized to examine the ability of the mitochondria to synthesize ATP when provided with selected substrates. In any assay of ATP synthetic capacity, the amount of ATP produced in the absence of substrates is a result of the oxidation of endogenous substrates. Incubating cells with NAD-linked substrates, such as pyruvate, malate and glutamate, can assess ATP synthesis through the entire respiratory chain. Succinate, a FAD-linked substrate, with the addition of rotenone (a complex I inhibitor), bypasses the activity of complex I and can assess ATP synthesis through complexes II to V. The substrates tetramethylphenylenediamine (TMPD) and ascorbate reduce cytochrome *c* directly, and thus measure the flux through complex IV (Hofhaus et al.

1996). ATP synthesis was determined fluorometrically with hexokinase and glucose-6-phosphate dehydrogenase (Williamson et al. 1973).

At baseline, all twelve patients in this study, each with a confirmed OXPHOS disorder, had decreased ATP synthesis in lymphocytes with all substrates tested when compared to control subjects. There was a 34% reduction in ATP synthesis when no substrate was added, reflecting a reduced ability to oxidize endogenous substrates. The most marked reduction in ATP synthesis was observed with NAD-linked substrates (42% to 47%). The activity of the respiratory chain through complexes II to V was reduced by 36%. ATP synthesis through complex IV was decreased by 32% relative to control values. In contrast, after twelve months of treatment, the ATP synthesis reflecting the oxidation of endogenous substrates was only reduced by 12% relative to mean control values. The activity across the entire respiratory chain (using NAD-linked substrates), was reduced by 21% to 24%. ATP synthesis through complexes II to V was 21% below control values, while the activity through complex IV was decreased by 15% (Table 5.3). Thus, in striking contrast to the ATP synthetic capacity at the outset of this study, the ATP synthesis in the lymphocytes of the patients after twelve months of cofactor treatment reached the range of levels (± 2 SD) observed for the majority of the control subjects (Figure 5.2).

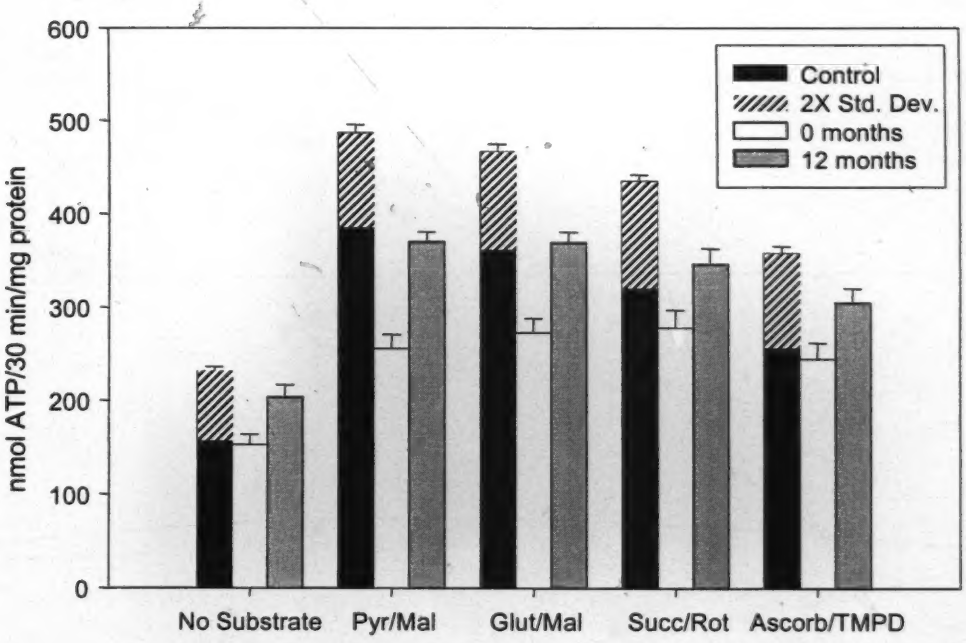

Figure 5.2 ATP Synthesis in Control Subjects and Patients at Baseline and after 12 months of treatment
Results are expressed as mean ± SEM.

Table 5.3 Comparison of ATP synthesis in Control Subjects and Patients*

Subjects	No Substrate	Pyruvate/ Malate	Glutamate/ Malate	Succinate/ Rotenone	Ascorbate/ TMPD
Control [a]	**231 ± 11** *n=62*	**487 ± 19** *n=63*	**466 ± 16** *n=57*	**435 ± 14** *n=56*	**357 ± 16** *n=56*
Patients [b]					
Baseline	**153 ± 20 (34)** *n=41*	**256 ± 27 (47)** *n=39*	**272 ± 27 (42)** *n=39*	**278 ± 33 (36)** *n=36*	**244 ± 29 (32)** *n=36*
12 Months	**204 ± 20 (12)** *n=28*	**370 ± 15 (24)** *n=28*	**369 ± 17 (21)** *n=29*	**346 ± 26 (21)** *n=27*	**305 ± 22 (15)** *n=26*

( ) = % decrease from mean control value
n = number of determinations
a = 14 subjects b = 12 patients
Values expressed as mean ± SEM
*nmol ATP/30min/mg protein
SEM calculated on number of control subjects (14) and number of patients (12).

ATP synthesis in the lymphocytes of patients at three, six and twelve months was expressed as a percentage increase of the mean value of baseline activity. After three months of treatment, there was an increase in ATP synthesis with all substrates tested, ranging from 112% to 122%. After twelve months of treatment, the increase ranged from 124% to 142%. The increase in ATP synthesis reached significance after three months of treatment, and ATP synthesis was significantly higher at twelve months as compared to three-month values. There was no significant difference in ATP synthesis between three and six months, or between six and twelve months (Figure 5.3).

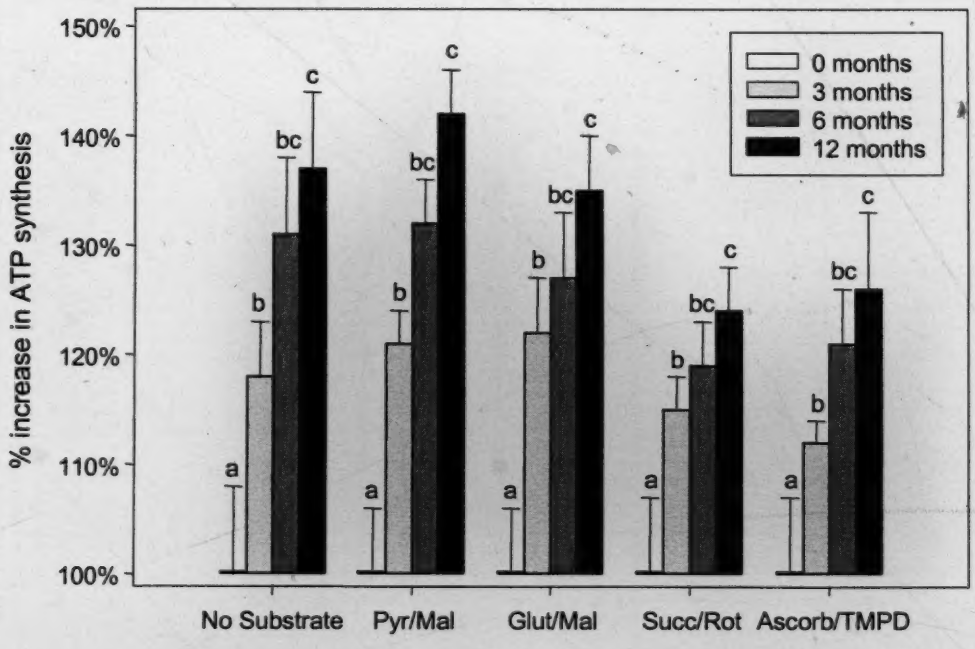

Figure 5.3 Effect of Cofactor Treatment on ATP Synthesis in Patients The results are expressed as a percentage increase of the mean value of baseline activity. Data represent all twelve patients with a minimum of two determinations from each time point. Values with different superscripts are significantly different ($p \leq 0.05$).

The effect of cofactor treatment in each of the five distinct mitochondrial disorders was also evaluated separately. In the six patients with the 11778 LHON mutation, the most marked increase in ATP synthesis after 12 months of treatment was noted with NAD-linked substrates (41% to 44%), which reflects respiratory chain activity through complex I. The 11778 mutation causes an amino acid substitution in the ND4 subunit of complex I, which is postulated to alter the interaction of complex I with ubiquinone (Degli Esposti et al. 1994). The oxidation of endogenous substrates increased by 33%, activity through complexes II to V increased by 19% and oxidation through complex IV increased by 23% (Figure 5.4; Table 5.4).

There was also an increase in ATP synthesis (21% to 36%) in the three patients with CPEO after 12 months of treatment. The increase was not markedly different for any of the substrates tested (Figure 5.5; Table 5.5) The deletion in the muscle tissue of these patients spans from the ATPase 8 gene to the ND5 gene and affects the functioning of complexes I, III, IV and V.

In the patient with the MELAS mutation, there was a 50% increase in the oxidation of endogenous substrates and a 46% to 54% increase in ATP synthesis using NAD-linked substrates after cofactor therapy. The increase in ATP synthetic capacity through complexes II to V, and through complex IV, was 21% (Figure 5.6; Table 5.6). A specific enzyme defect has not been identified in this patient, although deficiencies in complexes

I, III and IV, alone or in combination, have been reported in other cases of MELAS (Goto et al. 1992; Koo et al. 1993; Morgan-Hughes et al. 1995).

Biochemical analysis of the NARP mutation in fibroblasts and lymphoblasts has shown that ATP synthesis is decreased with all substrates (Tatuch and Robinson 1993; Makela-Bengs et al. 1995). In addition to the decrease in ATP synthesis with all substrates tested in the patient with the NARP mutation, the increase in ATP synthesis after a year of cofactor treatment appears universal with the substrates tested. There was a 44% increase in the oxidation of endogenous substrates, and ATP synthesis through complex IV increased by 36%, through complexes II to V by 34%, and through complex I by 23% to 28% (Figure 5.7; Table 5.7).

Complex IV is considered to be the rate-limiting enzyme in the respiratory chain (Robinson et al. 1985). A defect in complex IV has been postulated to prevent the generation of the mitochondrial membrane potential and the subsequent assembly of the respiratory chain components, resulting in a generalized deficiency of the respiratory chain (Attardi and Schatz 1988). In the patient with an isolated COX deficiency, ATP synthetic capacity before treatment was reduced by 63% to 75% relative to control values. This patient demonstrated the most marked increase in ATP synthesis after cofactor treatment: 84% to 128% with NAD-linked substrates, 54% through complexes II to V, 59% through complex IV, and 14% with oxidation of endogenous substrates (Figure 5.8; Table 5.8).

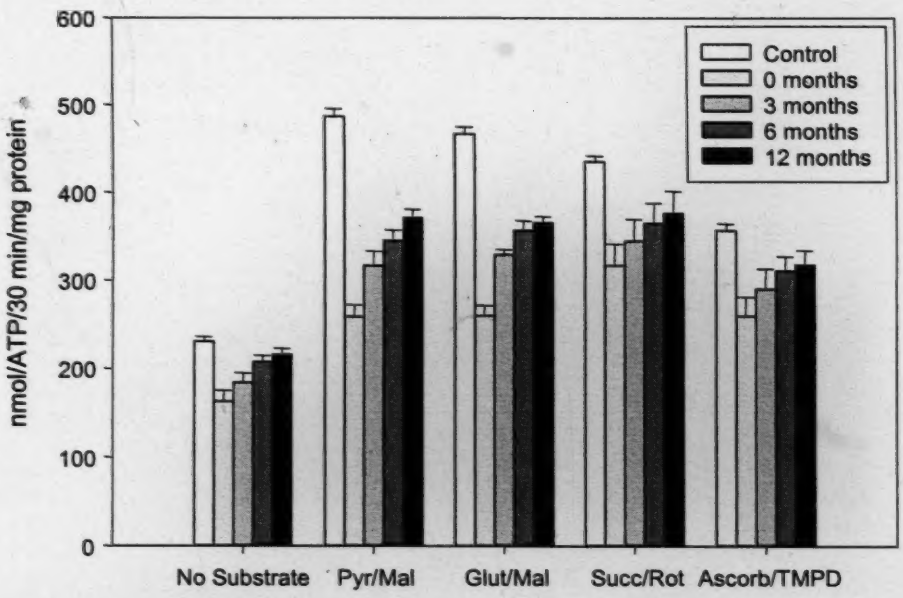

Figure 5.4 Effect of Cofactor Treatment on ATP Synthesis in LHON Patients
Results are expressed as mean ± SEM.

Table 5.4 Effect of Cofactor Treatment on ATP Synthesis in LHON Patients*

	No Substrate	Pyruvate/ Malate	Glutamate/ Malate	Succinate/ Rotenone	Ascorbate/ TMPD
0 months	**163 ± 24** *n=24*	**259 ± 26** *n=23*	**260 ± 21** *n=22*	**317 ± 47** *n=21*	**259 ± 41** *n=21*
3 months	**184 ± 17 (14)** *n=14*	**317 ± 26 (22)** *n=14*	**330 ± 9 (27)** *n=14*	**345 ± 37 (9)** *n=14*	**290 ± 35 (12)** *n=14*
6 Months	**208 ± 11 (28)** *n=14*	**345 ± 18 (33)** *n=14*	**357 ± 17 (37)** *n=14*	**365 ± 34 (15)** *n=13*	**310 ± 26 (20)** *n=14*
12 Months	**216 ± 11 (33)** *n=16*	**372 ± 14 (43)** *n=15*	**366 ± 11 (41)** *n=14*	**376 ± 40 (19)** *n=15*	**318 ± 25 (23)** *n=14*

( ) = % increase from mean control value
n = number of determinations
Data from 6 patients
SEM calculated on number of patients (6).

Values expressed as mean ± SEM
*nmol ATP/30min/mg protein

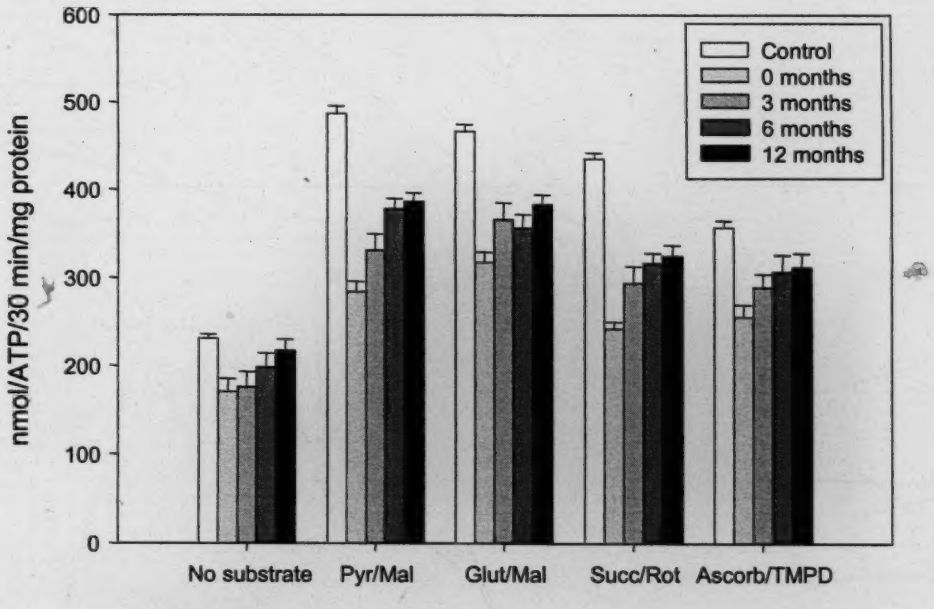

Figure 5.5 Effect of Cofactor Treatment on ATP Synthesis in CPEO Patients
Results are expressed as mean ± SEM.

Table 5.5 Effect of Cofactor Treatment on ATP Synthesis in CPEO Patients*

	No Substrate	Pyruvate/ Malate	Glutamate/ Malate	Succinate/ Rotenone	Ascorbate/ TMPD
0 months	**171 ± 24** *n=8*	**284 ± 18** *n=8*	**317 ± 20** *n=8*	**242 ± 13** *n=8*	**255 ± 21** *n=7*
3 months	**176 ± 24 (3)** *n=6*	**331 ± 27 (17)** *n=6*	**366 ± 27 (15)** *n=6*	**294 ± 25 (21)** *n=6*	**289 ± 24 (13)** *n=8*
6 Months	**198 ± 23 (16)** *n=6*	**378 ± 17 (33)** *n=6*	**356 ± 23 (12)** *n=6*	**316 ± 17 (31)** *n=7*	**306 ± 27 (20)** *n=6*
12 Months	**217 ± 18 (27)** *n=6*	**386 ± 14 (36)** *n=6*	**383 ± 16 (21)** *n=6*	**324 ± 17 (34)** *n=6*	**311 ± 23 (22)** *n=6*

( ) = % increase from mean control value
n = number of determinations
Data from 3 patients
SEM calculated on number of patients (3).

Values expressed as mean ± SEM
*nmol ATP/30min/mg protein

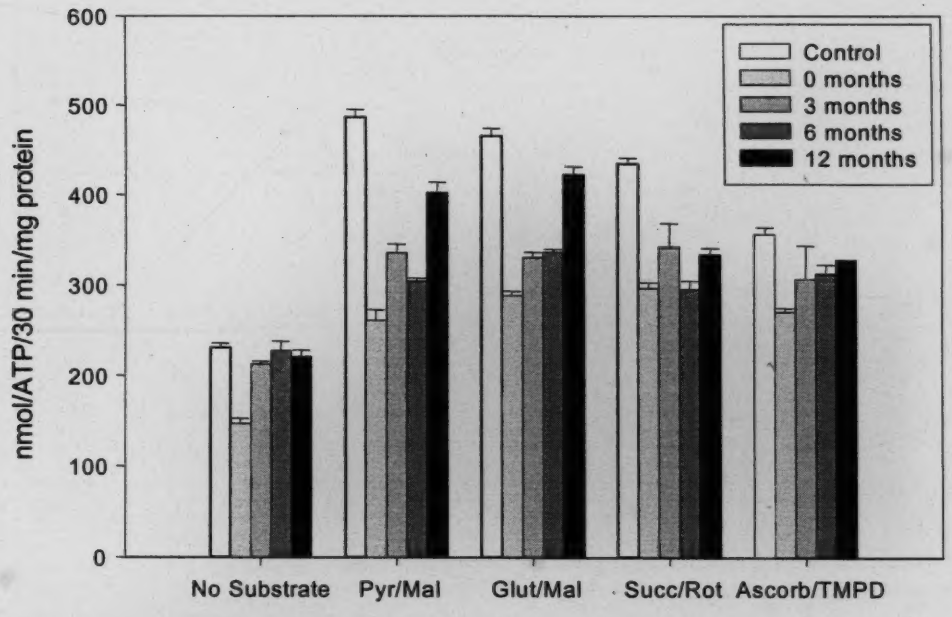

Figure 5.6 Effect of Cofactor Treatment on ATP Synthesis in the MELAS Patient
Results are expressed as mean ± SEM.

Table 5.6 Effect of Cofactor Treatment on ATP Synthesis in the MELAS Patient*

	No Substrate	Pyruvate/ Malate	Glutamate/ Malate	Succinate/ Rotenone	Ascorbate/ TMPD
0 months	**147 ± 6** *n=4*	**261 ± 12** *n=4*	**289 ± 5** *n=4*	**297 ± 6** *n=4*	**271 ± 4** *n=2*
3 months	**213 ± 3 (44)** *n=2*	**336 ± 10 (29)** *n=2*	**331 ± 6 (15)** *n=2*	**343 ± 26 (15)** *n=2*	**307 ± 37 (14)** *n=2*
6 months	**228 ± 11 (55)** *n=2*	**305 ± 4 (17)** *n=2*	**337 ± 3 (17)** *n=2*	**296 ±9** *n=2*	**313 ± 10 (15)** *n=2*
12 Months	**221 ± 7 (50)** *n=2*	**403 ± 11 (54)** *n=2*	**423 ± 9 (46)** *n=2*	**333 ± 8 (21)** *n=2*	**328 ± 0 (21)** *n=2*

( ) = % increase from mean control value
n = number of determinations
SEM calculated on number of determinations, given only one patient.
Values expressed as mean ± SEM
*nmol ATP/30min/mg protein

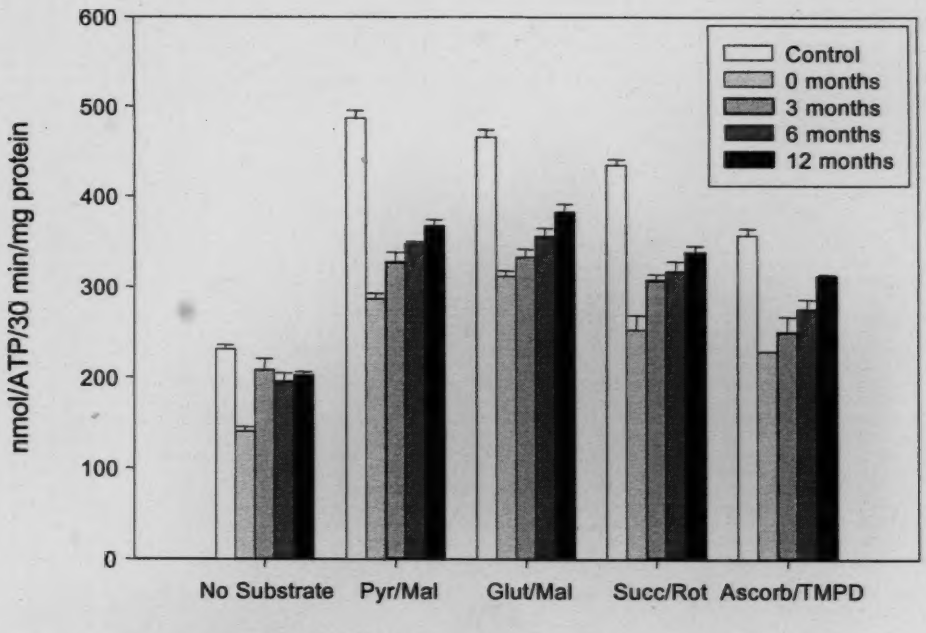

Figure 5.7 Effect of Cofactor Treatment on ATP Synthesis in the NARP Patient
Results are expressed as mean ± SEM.

Table 5.7 Effect of Cofactor Treatment on ATP Synthesis in the NARP Patient*

	No Substrate	Pyruvate/ Malate	Glutamate/ Malate	Succinate/ Rotenone	Ascorbate/ TMPD
0 months	141 ± 5 *n=2*	287 ± 6 *n=2*	312 ± 6 *n=2*	253 ± 17 *n=2*	229 ± 0 *n=2*
3 months	208 ± 23 (48) *n=2*	327 ± 11 (14) *n=2*	333 ± 8 (7) *n=2*	308 ± 6 (22) *n=2*	250 ± 17 (9) *n=2*
6 months	196 ± 9 (39) *n=2*	348 ± 2 (21) *n=2*	356 ± 9 (14) *n=2*	318 ± 11 (26) *n=2*	276 ± 11 (21) *n=2*
12 Months	203 ± 4 (44) *n=2*	367 ± 7 (28) *n=2*	383 ± 9 (23) *n=2*	338 ± 7 (34) *n=2*	312 ± 2 (36) *n=2*

( ) = % increase from mean control value
n = number of determinations
SEM calculated on number of determinations, given only one patient.
Values expressed as mean ± SEM
*nmol ATP/30min/mg protein

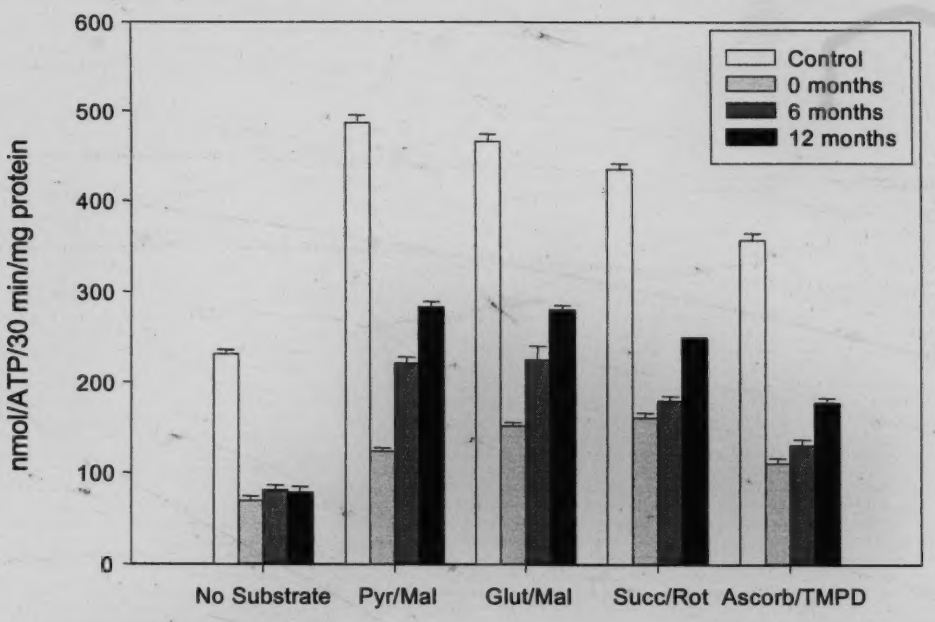

Figure 5.8 Effect of Cofactor Treatment on ATP Synthesis in the COX deficiency patient
Results are expressed as mean ± SEM.

Table 5.8 Effect of Cofactor Treatment on ATP Synthesis in the COX Deficiency Patient*

	No Substrate	Pyruvate/ Malate	Glutamate/ Malate	Succinate/ Rotenone	Ascorbate/ TMPD
0 months	**69 ± 5** *n=3*	**124 ± 4** *n=3*	**152 ± 4** *n=3*	**161 ± 6** *n=3*	**111 ± 6** *n=2*
6 months	**81 ± 6 (17)** *n=2*	**221 ± 7 (78)** *n=3*	**225 ± 15 (48)** *n=29*	**178 ± 5 (11)** *n=2*	**131 ± 6 (18)** *n=3*
12 Months	**79 ± 6 (14)** *n=2*	**283 ± 6 (128)** *n=3*	**280 ± 5 (84)** *n=3*	**249 ± 0 (54)** *n=2*	**177 ± 6 (59)** *n=2*

( ) = % increase from mean control value
n = number of determinations
Data not available for 3 months
SEM calculated on number of determinations, given only one patient.
Values expressed as mean ± SEM
*nmol ATP/30min/mg protein

DISCUSSION

The measurement of ATP synthesis in circulating lymphocytes provides an ideal tool for objectively evaluating therapy and monitoring progression of an OXPHOS disorder. In the present study, it has been demonstrated for the first time that an increase in ATP synthetic capacity in lymphocytes from patients with an OXPHOS disorder, results from cofactor administration.

Clinical trials of cofactor treatment in OXPHOS disorders are difficult to perform. There are limited numbers of patients with any one specific disorder, as well as great phenotypic variability within a specific genotype. It is difficult to reliably utilize clinical observation to assess the effectiveness of the treatment in an open study such as this. The reliability of the clinical assessment was further hampered in this study by the inability to have the same metabolic physician evaluate the patients at the three, six and twelve month time points. Clinical measurements are also complicated by the combination of irreversible tissue degeneration and potentially reversible cellular changes caused by the OXPHOS defect. In patients with the LHON 11778 mutation, the degeneration of the optic nerve and retinal ganglion cell layer which cause central vision loss is generally irreversible (Huoponen 2001; Wallace et al. 2001). Although visual recovery is rare in affected individuals who carry the 11778 mutation (4%), approximately half of all 14484 LHON patients show improvement after the initial vision loss. One possible explanation for the

differences observed in vision recovery among the LHON mutations is that they differ in the levels of free radicals that are produced (Howell 1997). It is not known whether cofactor treatment would result in the slowing of oxidative damage and enhance possible vision recovery in these patients. Reversible cellular changes in post-mitotic tissues such as muscle have been demonstrated by the regeneration of satellite cells, which are incorporated into existing muscle fibers. A re-biopsy of muscle tissue of a patient with a heteroplasmic tRNA point mutation showed that the regenerating muscle fibers were essentially homoplasmic for wild-type mtDNA, while the non-regenerating fibers contained predominantly mutant mtDNA (Shoubridge et al. 1997). Methods promoting satellite cell incorporation into existing myofibers may enhance muscle function. Aerobic training has also been found to improve exercise tolerance and oxidative capacity in patients with mitochondrial myopathies (Taivassalo et al. 2001). The cellular basis of the increased oxygen utilization was thought to be training-induced mitochondrial proliferation, which in turn may have improved oxidative phosphorylation, although there was no preferential proliferation of the wild-type mtDNA. One of the goals of cofactor treatment is to improve ATP synthesis, thus reversing some of the damage caused by decreased cellular energy. It is hoped that by increasing ATP production, there will be a reduction in the oxidative stress caused by the impairment in the respiratory chain. The excessive production of oxygen radicals is known to accelerate the already high

mutational rate of mtDNA; further mutation and impairment of ATP generation thus becomes a self-perpetuating process (Ames et al. 1995). Treatment is unlikely to reverse existing damage; the most likely response is a slowing in the rate of deterioration, which will only become apparent after prolonged treatment.

The mean duration of the disease in this study population was 14 years, so the lack of obvious clinical improvement after a one-year treatment period is not surprising. It is not known whether prolonged cofactor treatment will be effective in improving the clinical symptoms of these patients. Other controlled trials have failed to demonstrate a clearly beneficial effect of combined cofactor treatment on the entire study population in terms of clinical improvement (Matthews et al. 1993; Peterson 1995). The findings in this study are supported by other studies in which treatment with vitamin cofactors and electron acceptors improved metabolic parameters, but failed to show an amelioration of clinical symptoms (Bresolin et al. 1988; Chan et al. 1998). Any OXPHOS disorder may affect different organ systems in each affected individual, making outcome measurements difficult to assess. It may also not be reasonable to expect that a single treatment regime would have a similar effect on all OXPHOS disorders. Exacerbations and remissions are also characteristic of these disorders, further complicating the evaluation of the efficacy of the treatment.

One of the promising areas for cofactor treatment may be the possible prevention of associated clinical symptoms in patients who are diagnosed early or have milder forms of the disease. This approach to preventing progression of the disease relates to heteroplasmy and the threshold effect. The severity of the OXPHOS defect resulting from mutated mtDNA is a function of the proportion of mutant mtDNA, compared to wild-type, and of the differing energy requirements of the various organs and tissues (Wallace 1992). This is referred to as "threshold expression" and reflects the reliance of each organ or tissue on mitochondrial energy production. It is postulated that in mild cases of OXPHOS disorders, slight improvements in ATP synthesis could diminish the threshold for disease expression and prevent the progression of clinical symptoms. The most likely clinical response would be a decrease in the rate of deterioration. Large, multi-center trials will be necessary to determine whether prolonged cofactor treatment can decrease morbidity or alter the clinical course of these diverse conditions.

Plasma or serum CoQ_{10} values in human subjects vary widely in healthy individuals. In an investigation of serum CoQ_{10} levels in healthy subjects, the average CoQ_{10} level was 1.36 µg/mL with concentrations ranging from 0.57 to 3.03 µg/mL. The confidence interval of 95% was 1.21 to 1.50 µg/mL (Laaksonen et al. 1995). The mean pre-treatment serum CoQ_{10} value of the patients in this study was 0.62 ± 0.07 µg/mL. Although this value is within the range of normal control values, it is below the

reported 95% confidence interval. It is postulated that OXPHOS deficiency could lead to an increased consumption of CoQ_{10} and therefore contribute to the lower CoQ_{10} levels (Littarru and Battino 2000). *In vitro* studies have indicated that electron transfer, and hence the efficiency of oxidative phosphorylation, may be limited by the concentration of CoQ_{10}, which at physiological levels is not saturating for maximal electron transfer (Lenaz et al. 1994; Lenaz et al. 1997). It has been proposed that CoQ_{10} levels above normal could counteract the decreased rate of respiratory chain enzyme activity by enhancement of NADH oxidation (Lenaz et al. 1997). It is not known why serum CoQ_{10} in the subjects did not reach a maximum level after three months of treatment and instead continued to increase up to twelve months. It is possible that continued supplementation may cause an induction of enzymes that enhance absorption or delivery of CoQ_{10} to peripheral tissues. Because the patients in this study were consuming a combination of cofactors, it is only speculative that the increase in ATP synthesis is related to the elevated plasma concentration of CoQ_{10}.

Regardless of the lack of a clear improvement in clinical symptoms, a significant increase in ATP synthetic capacity was demonstrated in the lymphocytes of the patients with OXPHOS disorders who were treated with cofactor therapy. Impaired activity of the respiratory chain enzyme complexes decreases respiration and lowers proton pumping, decreasing the membrane potential and the proton-motive force across the mitochondrial inner membrane (Kobayashi et al. 1991; James et al. 1996).

decrease in this gradient lowers the maximal rate of ATP synthesis. The improvement in ATP synthesis in lymphocytes demonstrated in this study may correspond to a slowing or arrest of the progressive deterioration normally observed in mitochondrial disorders.

In the combined cofactor treatment, it is difficult to determine the effects of the individual components of the cofactor treatment on ATP synthesis, and which cofactors may prove to be beneficial remains to be determined. In order to further elucidate the mechanisms of action of cofactor treatment, the effect of each cofactor needs to be evaluated by incubation of control cells with the individual component and monitoring of ATP production.

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CHAPTER 6

EFFECT OF COFACTOR INCUBATION ON ATP SYNTHETIC CAPACITY IN CONTROL LYMPHOCYTES

INTRODUCTION

The effect of a therapeutic regimen of mitochondrial cofactors and antioxidants in patients with OXPHOS disorders was assessed by analyzing ATP synthesis in circulating lymphocytes (Chapter 5). A statistically significant increase in ATP synthetic capacity after cofactor treatment was demonstrated. These results mark the first time that an improvement in the ability to synthesize ATP has been demonstrated in lymphocytes from mitochondrial patients being treated with combined cofactor therapy. What remains unclear from these results is which of the supplemented cofactors are responsible for the improved synthesis of ATP. The effect of the individual components of the cofactor regimen and their dosages in the treatment of OXPHOS disorders remains to be determined. Examination of the effect of individual cofactors at a biochemical level will help to refine and improve the current therapeutic protocols.

The present study was designed to assess the effects of individual cofactors on mitochondrial ATP synthetic capacity, as measured in circulating lymphocytes. ATP synthetic capacity upon *in vitro* cofactor supplementation was assessed by treating isolated lymphocytes from control subjects with each cofactor individually. The data from this study demonstrate that there is an increase in ATP synthesis upon CoQ_{10}

incubation of control lymphocytes. These results may have implications for the treatment of patients with OXPHOS disorders and suggest that CoQ_{10} may be the active ingredient in the current treatment protocol.

MATERIALS AND METHODS

Blood samples were collected from healthy control subjects (after obtaining informed consent) and lymphocytes were isolated from the heparinized sample as described (Fotino et al. 1971). The lymphocyte pellet was re-suspended in medium containing 50% modified Eagle's medium (MEM), 40% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO), then stored at -80°C. The viability of these lymphocytes is approximately 85%; previously frozen lymphocytes thus serve as an appropriate tissue for testing the ability of individual cofactors to affect ATP synthesis. Studies of ATP synthesis were performed as previously described (Chapter 4). The cells were incubated with two different substrate combinations (pyruvate/malate and succinate/rotenone), in the presence or absence of the individual cofactors. Pyruvate/malate was used to assess the efficacy of ATP synthesis through the entire respiratory chain, while succinate/rotenone was used to assess the efficacy of ATP synthesis through complexes II to V. ATP synthesis was measured by standard fluorometric methods (Williamson and Corkey 1969).

The amount of cofactor used in the incubation medium was proportional to the amount consumed by the patients (cofactor amount per µg cellular protein and oral dosages are listed in Table 6.1).

Table 6.1 Comparison of Oral Supplementation and Incubation Dosages

	Oral Supplementation (Daily Dosage)	**Incubation Dosage** (µg per µg protein)
Coenzyme Q_{10}*	5 mg/kg (350mg)**	0.01 – 2.0
Carnitine	500 mg/day	1.0 - 30
Vitamin B Complex***		
Thiamine	25 mg	0.2 – 2.0
Riboflavin	25 mg	0.2 – 2.0
Niacinamide	25 mg	0.2 – 2.0
Pyridoxine	25 mg	0.2 – 2.0
Pantothenic Acid	25 mg	0.2 – 2.0
Biotin	25 µg	0.002 – 0.02
Cyanocobalamine	25 µg	0.002 – 0.02
Folic Acid	1 mg	0.008 – 0.08
Vitamin C	1000 mg	8.0 – 80.0
Vitamin K_1 (Phylloquinone)	0.4 mg/kg (28 mg)**	0.2 – 2.0

* Adjustments were made to the starting dose as a response was obtained at the intial starting dose of 0.2 µg.
** Dosage based on a 70 kg individual.
*** Incubation mixture was solubilized in 20 mM Tris-HCl, pH 7.4.

The quantity of the individual cofactor was titrated, using incremental increases in concentration, until maximal stimulation of ATP synthesis was achieved. Once the optimal level of ATP stimulation was achieved, cells were incubated from five to 240 minutes. Time course studies were not carried out for cofactors that did not show a significant dose response.

Liposome Preparation of Coenzyme Q_{10}

Because CoQ_{10} is highly lipophilic, supplementation in the assay was accomplished by incorporation of CoQ_{10} into liposomes. Soybean phospholipid (5 g) was mixed together with 18 mg coenzyme Q_{10} (Sigma®). The mixture was dissolved in 14 mL chloroform and evaporated to dryness under vacuum at 30°C for two hours using a Buchi Rotary evaporator (Schneider et al. 1982). The dry phospholipid-ubiquionone mixture was stored in sealed containers at -80°C until use. Liposomes were prepared by sonicating 0.2 g of the phospholipid-ubiquinone mixture in 1 mL of the digitonin incubation buffer with the microtip of a Branson sonicator (Model 450) at 40 W, using three 10-second cycles. Measured amounts of liposomes (2.8 µg to 560 µg liposomes providing 0.01 µg to 2 µg CoQ_{10}) were added to the lymphocyte mixture and measurement of ATP synthesis was performed as described.

RESULTS

Effects of CoQ_{10} Incubation on ATP Synthetic Capacity

In the absence of CoQ_{10}, the amount of ATP produced by lymphocytes with the NAD-linked substrates (pyruvate/malate) was 437 ± 19

nmol/30min/mg protein. Similarly, 439 ± 18 nmol/30min/mg protein was synthesized with succinate/rotenone (which measures the activity through complexes II to V). Addition of CoQ_{10} to the incubation caused an increase in ATP synthesis, with the pattern of increase in ATP production similar for both substrates. Linear increases in ATP synthesis were observed with CoQ_{10} dosages of 0.01 to 0.1 µg CoQ_{10}/µg protein and 0.6 to 1.3 µg CoQ_{10}/µg protein, suggestive of a biphasic response to concentration. Maximal stimulation in ATP synthesis (approximately 200%) was observed at 1.5 µg CoQ_{10}/µg protein (1348 ± 9 nmol/30min/mg protein for pyruvate/ malate and 1349 ± 8 nmol/30min/mg protein for succinate/rotenone). No further increase in ATP synthesis was seen with dosages of 1.5 to 2.0 µg CoQ_{10}/µg protein (Figure 6.1).

Time Course of CoQ_{10} Incubation on ATP synthesis

To determine the time course for the effect of CoQ_{10} on ATP synthesis, lymphocytes plus 2.0 µg CoQ_{10}/µg protein were assayed for total ATP synthesized. ATP synthesis was assessed every five minutes from five to 30 minutes, then every 30 minutes thereafter up to 240 minutes. Pyruvate/malate was the substrate mix used in all incubations as no differences were observed between pyruvate/malate and succinate/rotenone. A maximum amount of ATP appeared to be synthesized by 90 minutes (Figure 6.2). The time course for ATP synthesis in lymphocytes in the absence of CoQ_{10} shows a similar pattern to that seen in the presence of CoQ_{10} (data not shown). These data

indicate that the length of the incubation period is not significantly affected by the addition of CoQ_{10} and that maximal ATP synthesis is reached by 90 minutes.

Assessment of Non-Mitochondrial ATP synthesis

The measurement of ATP synthesis, using hexokinase and glucose-6-phosphate dehyrogenase, is assessed by examining the fluorescence of NADPH formed at 450 nm (excitation at 340 nm). The increase in fluorescence accompanying the conversion of glucose + ATP + $NADP^+$ to ADP + NADPH + H^+ gives a quantitative measurement of ATP. Mitochondrial ATP synthesis measurements using permeabilized fibroblasts have traditionally not evaluated the contribution of non-mitochondrial ATP production (Robinson et al. 1990; Wanders et al. 1993). In a permeabilized cell, the steady-state ATP concentration is a balance of both synthetic and degradation reactions (Ouhabi et al. 1998). Potential sources of ATP synthesis include the glycolytic pathway and conversion of cyclic AMP and inorganic phosphate to ATP by adenylate kinase. ATP hydrolysis is dependent on cytosolic magnesium, which is blocked in the assay system by the presence of EDTA in the incubation medium. Although most cytosolic coenzymes and metabolites may be lost upon cell permeabilization, some remain. In addition, as this is an indirect assay it is difficult to assess the effect of membrane-associated NADH oxidoreductases on increases in fluorescence seen in this assay system. Oligomycin is known to inhibit the membrane-bound mitochondrial

ATPase through binding to the membrane sector of the enzyme; addition of this antibiotic stops mitochondrial ATP production (James et al. 1999). Non-mitochondrial ATP synthesis was determined by addition of 20 µg/ml oligomycin to the incubation medium; the rate of mitochondrial ATP synthesis was determined by the difference between the assays in the presence or absence of oligomycin. Mitochondrial ATP synthesis in lymphocytes was initially assessed by incubating cells with pyruvate/malate in the absence of CoQ_{10}. The apparent ATP synthesis with these substrates was 472 ± 7 nmol/30min/mg protein, whereas in the presence of oligomycin, 218 ± 4 nmol of ATP appeared to be synthesized. Therefore, approximately 46% of the ATP produced is attributed to non-mitochondrial sources and non-specific NADH/NADPH oxidation. This suggested that the amount of ATP produced through oxidative phosphorylation is approximately 254 nmol/30min/mg protein. Upon incubation of lymphocytes with 2 µg CoQ_{10}/µg protein and pyruvate/malate, total apparent ATP synthesis was 1362 ± 9 nmol/30min/mg protein, which was reduced to 983 ± 10 nmol/ 30min/mg protein with the addition of oligomycin. The amount of mitochondrial ATP produced, and attributable to CoQ_{10} addition, was thus 379 nmol/30min/mg protein. This demonstrates that 28% of the ATP produced with the CoQ_{10} is via the respiratory chain. To determine whether there was an effect of the phospholipid vesicles on the assay, lymphocytes were incubated with liposomes that did not contain CoQ_{10}. A 35% increase in

ATP synthesis was observed with the phospholipid-containing liposomes. It is interesting to note that blocking mitochondrial ATP production with oligomycin did not have an effect, thus attributing the increase in ATP synthesis upon unoccupied liposome incubation completely to non-mitochondrial production. These results demonstrate a 49% increase in mitochondrial ATP synthesis, solely attributed to the addition of CoQ_{10} (Figure 6.3).

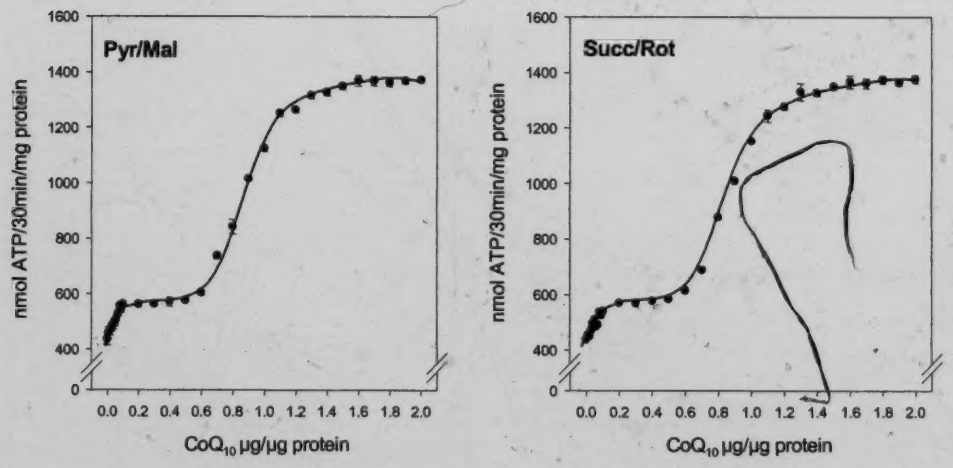

Figure 6.1 Effect of CoQ_{10} Incubation on ATP Synthetic Capacity
Values are the mean ± SEM of 2 independent samples with 2 determinations for each sample.

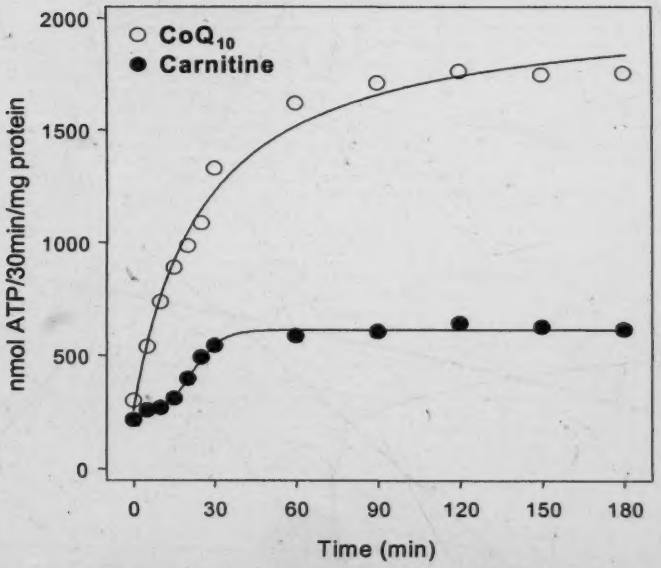

Figure 6.2 Time Course of CoQ_{10} and Carnitine Incubation on ATP Synthesis

Values are the mean ± SEM of 3 independent samples with 2 determinations for each sample.

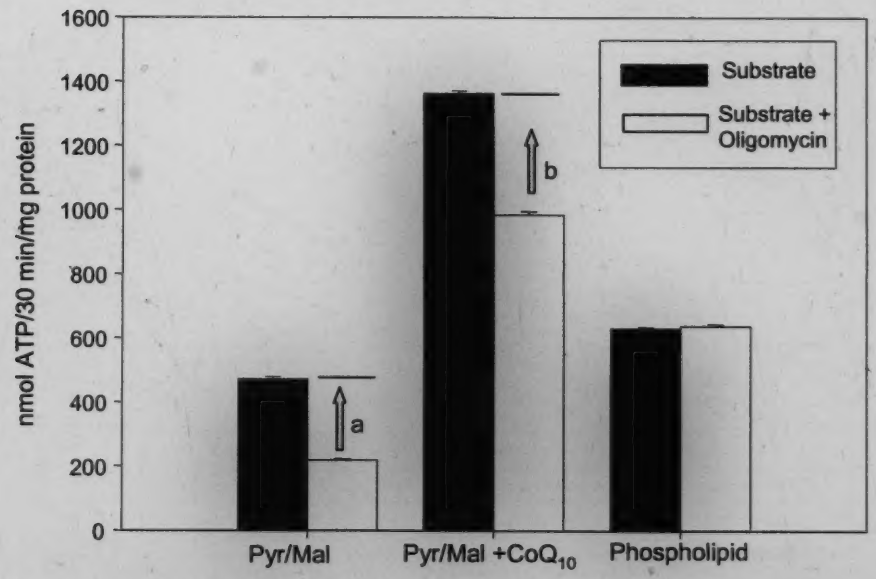

Figure 6.3 Effect of Blocking ATP Synthesis with Oligomycin

Values are the mean ± SEM of 5 independent samples with 2 determinations for each sample.

a = Mitochondrial ATP Synthesis ~ 254 nmol

b = Mitochondrial ATP Synthesis ~ 379 nmol

Effects of Carnitine Incubation on ATP Synthetic Capacity

The starting dose of carnitine was 1 µg/µg protein and this was increased by increments of 5 µg carnitine/µg protein to a maximum dosage of 30 µg carnitine/µg protein (Figure 6.4). The larger dosages were assayed for their effect on ATP synthesis as clinical improvement has been observed in patients with doses ranging from 2 g/day to 200 mg/kg/day (Bernsen et al. 1991; Campos et al. 1993; Scholte et al. 1995; Ogle et al. 1997). Although a significant increase had not been observed with the carnitine incubation, a time course similar to that used for CoQ_{10} was performed for comparative purposes, demonstrating that maximal ATP synthesis is reached by 90 minutes (Figure 6.2).

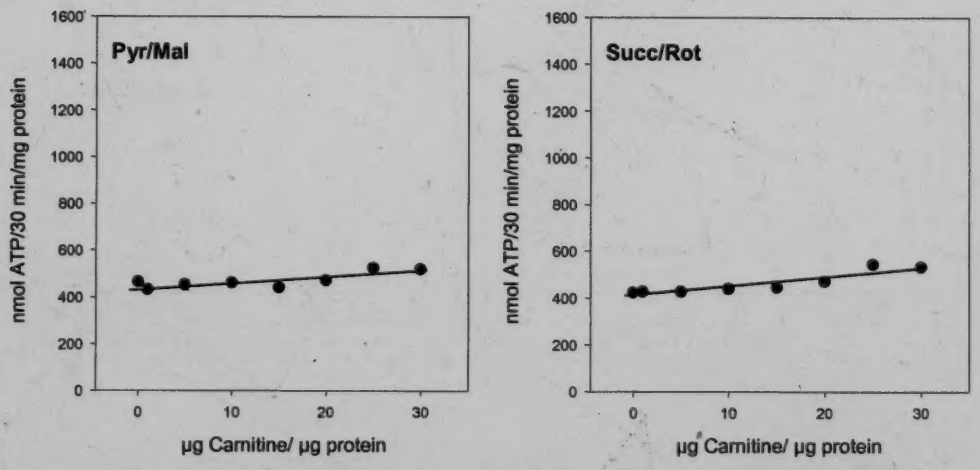

Figure 6.4 Effect of Carnitine Incubation on ATP Synthetic Capacity
Values are the mean ± SEM of 3 independent samples with 2 determinations for each sample.

Effects of Vitamin B Complex Incubation on ATP Synthetic Capacity

The starting dose of the vitamin B mixture was 8 µL/µg protein and this was increased by 8 µl increments up to 80 µL/µg protein. There was no significant increase in ATP synthesis upon incubation of control lymphocytes with the vitamin B complex with either pyruvate/malate or succinate/rotenone (Figure 6.5). Because stimulation of ATP synthesis was not achieved with the combined mixture, the effects of incubating each of the components of the vitamin B complex separately were not assessed (with the exception of riboflavin). A recent study described a 2.5-fold increase in ATP synthesis when 5 µmol/L riboflavin was added to the culture medium of fibroblasts from a patient with a nuclear-encoded complex I deficiency (Bar-Meir et al. 2001). To determine whether there might be a specific response to riboflavin, control lymphocytes were incubated with pyruvate/malate plus 5 µmol/ L and 10 µmol/ L of riboflavin. An 11% and 18% (untreated lymphocytes – 450 ± 9 nmol/30/min/mg protein; plus 5 µmol/L of riboflavin – 499 ± 6 nmol/30/min/mg protein; plus 10 µmol/L of riboflavin – 531 ± 4 nmol/30/min/mg protein) increase in ATP synthesis, respectively, was observed. This increase was completely blocked by the addition of 20 µg/mL oligomycin (data not shown). The increase in ATP synthesis observed was therefore attributed to non-mitochondrial ATP production or other non-specific reduction reactions.

Effects of Vitamin K Incubation on ATP Synthetic Capacity

Vitamin K_1 (phylloquinone) was incubated with control lymphocytes starting at 0.2 µg/ µg protein and increased in 0.2 µg increments to a final dosage of 2 µg/ µg protein. There was no significant increase in ATP synthesis by adding vitamin K_1 to the lymphocyte incubation with either pyruvate/malate or succinate/rotenone (Figure 6.6).

Effects of Combined Cofactor Incubation on ATP Synthetic Capacity

To assess whether there might be a synergistic response elicited by the combined cofactor treatment, control lymphocytes were incubated with the following mixture: 2.0 µg CoQ_{10}, 30 µg carnitine, 80 µL vitamin B complex and 2 µg vitamin K_1/µg protein. ATP synthesis was assessed using only pyruvate/malate. The ATP synthesis with the combined cofactor treatment (1348 ±12 nmol/30min/mg protein) was not significantly different from that obtained with CoQ_{10} alone (1337 ± 10 nmol/30min/mg protein).

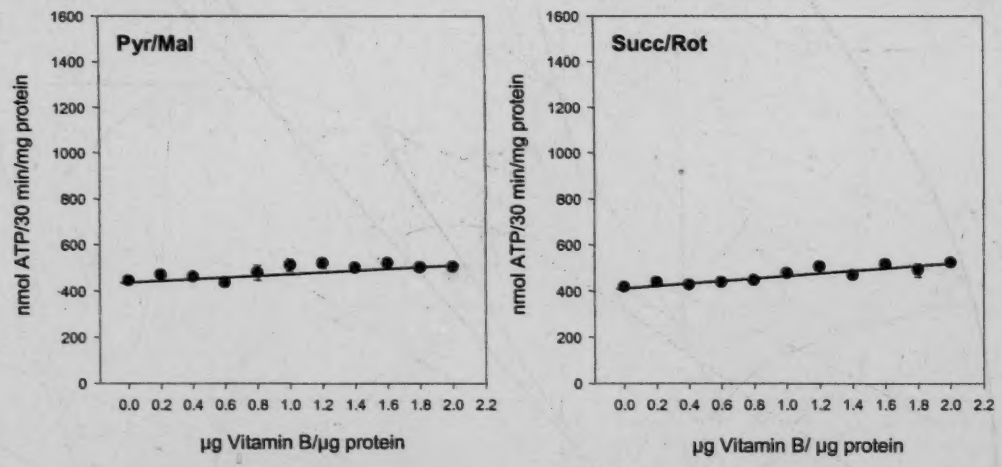

Figure 6.5 Effect of Vitamin B Complex Incubation on ATP Synthetic Capacity
Values are the mean ± SEM of 2 independent samples with 2 determinations for each sample.

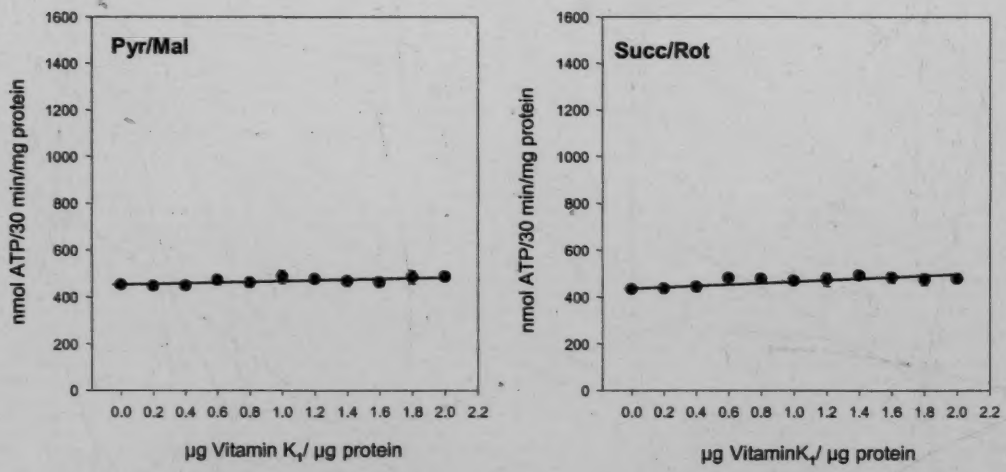

Figure 6.6 Effect of Vitamin K Incubation on ATP Synthetic Capacity
Values are the mean ± SEM of 2 independent samples with 2 determinations for each sample.

DISCUSSION

The incubation of control lymphocytes with individual cofactors demonstrated that ATP synthetic capacity increased in a dose-dependent manner with CoQ_{10} supplementation. The low solubility of CoQ_{10} in water necessitated the use of a lipophilic carrier to allow CoQ_{10} to permeate the lipid bilayers and accumulate in the mitochondrial membrane (Schneider et al. 1982). A biphasic increase in ATP synthesis with the CoQ_{10} incubation was observed in the present study. The incorporation of CoQ_{10} in mitochondrial membranes has previously been shown to exhibit an apparently biphasic behavior (Degli Esposti et al. 1981). It is not clear whether this is a result of the limited miscibility of CoQ_{10} with the phospholipid bilayers or a consequence of diffusion control of the CoQ_{10}.

Incorporation of phospholipids into the mitochondrial inner membrane had been found to cause an increase in the average distance between integral membrane proteins, and electron transfer in complexes I, II, and III decreased in proportion to the increase in the bilayer surface area. Because the decreased electron transfer rates correlated with the increased distances, it was initially concluded that there was a diffusion-limited step in the transfer of electrons between the complexes (Schneider et al. 1980). However, further comparisons of membranes enriched in phospholipids alone, or in phospholipid plus CoQ_{10}, showed a complete restoration of electron transfer activity in samples supplemented with CoQ_{10}, indicating that CoQ_{10} diffuses independently in the mitochondrial

membrane phospholipids (Schneider et al. 1982). Other investigators have demonstrated that by fusing phospholipids with an excess of CoQ_{10}, electron transfer was increased above the original level, suggesting that the concentration of CoQ_{10} in the membrane is the most important factor in regulating the rate of ATP synthesis (Battino et al. 1990). If CoQ_{10} diffuses randomly in the mitochondrial membrane, and the concentration of CoQ_{10} regulates the rate of electron transfer, why there is a non-linear relationship between the CoQ_{10} concentration and ATP synthesis is unclear. In the case of CoQ_{10}, the viscosity of the membrane and the crowding of the diffusion path by proteins are possible inhibitors of diffusion (Hackenbrock et al. 1986). The addition of exogenous CoQ_{10} enhances the respiratory turnover rate above physiological levels, while a decrease of CoQ_{10} content in the mitochondrial membrane lowers electron transfer in a reversible manner (Lenaz et al. 1993). Support for the response shown with CoQ_{10} incubation in the present experiments correlates with previous data demonstrating that CoQ_{10} concentration in the mitochondrial membrane is not physiologically saturating for maximal electron transfer (Battino et al. 1990; Lenaz et al. 1994). The maximal stimulation of ATP synthesis with CoQ_{10} observed in this study is thought to be a function of CoQ_{10} solubility in the membrane phospholipids.

In addition to an increase in mitochondrial ATP production with CoQ_{10}, a marked increase in non-mitochondrial ATP synthesis and non-specific NAD reduction was observed in the present investigations.

Although most of the non-mitochondrial ATP synthesis was attributed to the addition of the phospholipids alone, an increase in the non-mitochondrial ATP production was also noted when CoQ_{10} was added to the liposomes. This suggests additional functions for CoQ_{10} as a component of extra-mitochondrial redox reactions (Crane et al. 1993). A mechanism has been proposed in which enhanced availability of CoQ_{10}, as a result of exogenous supplementation, may increase NADH oxidation at the mitochondrial level as well as at the cytoplasmic level. CoQ_{10} would act as an electron acceptor for the plasma membrane-associated NADH dehyrogenases (Linnane et al. 1992). Several non-mitochondrial functions have been proposed for CoQ_{10}, including transplasma membrane electron transport that regulates the cytosolic NAD/NADH ratio. In cells treated with ethidium bromide to destroy the mtDNA, addition of CoQ_{10} activated the plasma membrane redox system, reoxidizing cystolic NADH and returning the NAD/NADH ratio toward normal levels (Martinus et al. 1993). It is not known whether this extra-mitochondrial role of CoQ_{10} would enable a patient with a respiratory chain disorder to maintain an adequate energy capacity to compensate for the decline in OXPHOS function.

In order for CoQ_{10} supplementation to have an impact in the treatment of OXPHOS disorders, it must be absorbed to a significant degree and incorporated into the mitochondrial membrane. Studies in rodents have shown that CoQ_{10} is incorporated into chylomicrons after intestinal absorption, transported by the lymphatic system to the

circulation, and subsequently incorporated into very-low-density-lipoproteins (VLDL) in the liver (Elmberger et al. 1989). Studies with human subjects have found that after oral administration of 200 mg of CoQ_{10}, the bulk was recovered in the triacylglycerol-rich (VLDL and chylomicrons) lipoprotein fractions within six hours of administration. In addition, CoQ_{10} was detected in all other lipoprotein fractions (Mohr et al. 1992). The efficacy of absorption and mitochondrial incorporation of CoQ_{10} has been studied in rodents, but limited information is available from human studies. It appears that the formulation, as well as the amount of the dose (due to the limited absorption), is important for efficient absorption and mitochondrial incorporation. Both animal and human studies have demonstrated that approximately 2% to 10% of the dose administered is taken up into the blood (Zhang et al. 1995; Weber 2001). CoQ_{10} is absorbed from supplements, as plasma CoQ_{10} levels increase significantly after oral administration. This finding was corroborated in these studies (Chapter 5), and supported by other investigators (Kaikkonen et al. 1997). The degree of mitochondrial incorporation is still unclear. An increase in the CoQ_{10} content in platelet and muscle mitochondria has been observed in human studies (Bresolin et al. 1988; Rotig et al. 2000). Oral CoQ_{10} administration in rats resulted in a significant increase in the cerebral cortex mitochondrial concentrations of CoQ_{10} (Matthews et al. 1998). The administration of CoQ_{10} remarkably improved the efficiency of brain and skeletal mitochondrial respiration in

six patients with different mitochondrial cytopathies, as assessed *in vivo* by ^{31}MRS (Barbiroli et al. 1997). These results are consistent with the view that an increased concentration of CoQ_{10} in the mitochondrial membrane improves oxidative metabolism. An increase in ATP synthetic capacity in control lymphocytes with CoQ_{10} administration, as demonstrated in this study, lends further support to the hypothesis that CoQ_{10} is incorporated into the mitochondria.

CoQ_{10} dosage in the treatment of mitochondrial disorders varies, but clinicians currently recommend 4 to 15 mg/kg/day (Gold and Cohen 2001). It is not known whether a substantial increase in oral CoQ_{10} supplementation in the patients in this study would have further enhanced the efficiency of oxidative phosphorylation. The present *in vitro* study demonstrates a maximal stimulation of ATP synthesis with incubation of 1.5 µg CoQ_{10}/µg protein, but it is difficult to extrapolate this finding to oral administration. Further studies are needed to determine efficacy in individual patients.

The incubation of control lymphocytes with vitamin B complex, carnitine and vitamin K_1 individually, or in combination did not result in an increase in ATP synthetic capacity. Successful therapy with single vitamins has often been due to a relative deficiency of the vitamin, or to a specific defect that utilizes the vitamin as a cofactor. Riboflavin is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which function as cofactors in complexes I and II. Riboflavin

responsiveness with clinical improvement has been described in several cases of complex I deficiency (Arts et al. 1983; Griebel et al. 1990; Bernsen et al. 1991; Penn et al. 1992; Bernsen et al. 1993; Scholte et al. 1995; Ogle et al. 1997). It is postulated that riboflavin may act by inhibiting the breakdown of complex I by stabilizing the complex in the membrane, with a subsequent increase in enzymatic activity (Vergani et al. 1999; Gold and Cohen 2001). Support for this hypothesis has been illustrated by an *in vitro* study, where a 2.5-fold increase in ATP synthesis in the fibroblasts of a patient with a complex I deficiency was observed with the addition of 5 µmol/L of riboflavin to the culture medium (Bar-Meir et al. 2001). In the same study, no change in ATP synthesis was shown in control fibroblasts treated with riboflavin. In control lymphocytes in this study, a significant increase in ATP synthetic capacity with the combined B vitamins or riboflavin alone was not observed. It is reasonable to assume that many of the cofactors will only be effective if a specific enzyme defect exists.

In the present study, the ability of various cofactors to stimulate ATP synthesis was examined, but whether the cofactors had any beneficial antioxidant effect was not evaluated. Antioxidants appear to delay clinical progression in various mouse models of mitochondrial disease (Wallace 1999). It may be that antioxidants such as CoQ_{10}, vitamin E, ascorbate and vitamin K may slow the process of oxidative damage, although the benefit over time is difficult to measure.

The data in this study suggest that CoQ_{10} may have a beneficial effect in the treatment of OXPHOS disorders. Of the cofactors tested, CoQ_{10} was the only compound that significantly increased ATP synthetic capacity in control lymphocytes. The evaluation of cofactor incubation in the lymphocytes of patients suffering from OXPHOS disorders will further enhance understanding in this area. Although there might be a best treatment regime for an individual patient with a specific OXPHOS disorder, it is unlikely that any one treatment will be appropriate for groups of patients suffering from mitochondrial diseases. The determination of ATP synthesis in patients with mitochondrial disorders on an individual basis may contribute to designing the most appropriate therapy on a disease-specific basis.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

The goal of this research was to test the hypothesis that there will be an increase in ATP synthesis in patients with oxidative phoshorylation disorders after cofactor treatment. A significant increase in ATP synthetic capacity in the lymphocytes of the twelve patients with OXPHOS disorders who were treated with nutritional cofactors was observed. Respiratory chain diseases represent an expanding group of clinically heterogeneous disorders associated with mutations in either the nuclear or mitochondrial genomes. The understanding of the mechanisms that contribute to the vast clinical and biochemical variability remains limited. Regardless of the mechanism, the end result is a decrease in energy production, resulting from impairment of the respiratory chain. The goal of nutritional cofactor treatment is to increase mitochondrial ATP production and thereby slow or arrest the progression of clinical symptoms.

It is not clear whether the improvement in ATP synthesis demonstrated in this study may correspond to a slowing or arrest of the progressive deterioration that is characteristic of these disorders. It is hoped that by increasing ATP production, the oxidative stress caused by the impaired respiratory chain will be reduced. The production of oxygen radicals by defective oxidative phosphorylation is known to increase the mutational rate of mtDNA; with subsequent impairment of ATP synthesis the process becomes a vicious cycle (Ames et al. 1995). Treatment is

unlikely to reverse existing damage; the most likely response to the cofactor therapy would be a slowing in the rate of deterioration, which will only become apparent after prolonged treatment. Longer-term studies will be required to determine whether cofactor treatment can alter the clinical course of these progressive disorders. With the advances in molecular diagnosis and the identification of mutations in nuclear genes, an increasing number of families with OXPHOS disorders are being recognized. Research on the potential disease-modifying effects of cofactor treatment in patients who can be identified early or in the pre-symptomatic stage of their illness is thus a priority. It is of utmost importance to determine whether pre-symptomatic treatment can reduce the morbidity associated with these disorders.

The patients in this study were all treated with the same combined cofactor treatment, regardless of the type of mitochondrial disorder. The underlying genetic and biochemical defects differed among our patients, and they exhibited a great variability of clinical symptoms. The mitochondrial disorders in this study represented a variety of disease states — ranging from isolated ocular disease to encephalopathy with neuromuscular involvement. It seems unlikely that a standard treatment would have a similar effect on all mitochondrial disorders, and thus studies of a standard treatment on a heterogeneous group of patients may not be applicable to an individual patient. In addition, developing a treatment trial examining the efficacy of cofactors by evaluating the response of all

possible organ systems would require an extremely large number of patients. Given the inherent problems mentioned above, and the difficulty of performing large clinical trials, individual trials where the patient serves as his or her own control may be a reasonable approach. The ability to utilize circulating lymphocytes as an objective tool for monitoring response to treatment and evaluating disease progression could provide important information to complement the clinical assessment of patients on specific treatment regimens.

The clinical assessment of patients with OXPHOS disorders is difficult due to the diverse nature of these diseases and the unpredictable clinical course. Exacerbations and remissions are also characteristic of these disorders, further complicating the evaluation of the efficacy of the treatment. Because of the varied range of clinical symptoms, it is difficult to determine which symptoms warrant monitoring in evaluating the effectiveness of cofactor therapy. The use of objective measurements such as nuclear magnetic resonance (NMR) spectroscopy would be an excellent addition in the evaluation of the clinical efficacy of these interventions. Phosphorus nuclear magnetic resonance (^{31} PNMR) can measure oxidative phosphorylation *in vivo,* quantitatively and non-invasively. ^{31}PNMR measures intracellular pH, inorganic phosphate (P_i), phosphocreatine (PCr), in addition to ATP levels. The resting phosphorylation potential (the ratio of PCr to P_i) is reduced in mitochondrial disorders, even in the absence of clinical symptoms

(Montagna et al. 1992). NMR spectroscopy can be used to investigate OXPHOS impairment and provide an objective *in vivo* method to evaluate effects of treatment intervention. NMR deserves further evaluation as an assessment tool in larger patient populations, because each patient can serve as their own control and the results of the intervention are objective. Unfortunately, attempts to secure funding to perform this aspect of the study were unsuccessful. Objective determination of the clinical status should be included in an effort to assess whether the biochemical improvement is mirrored by an improvement in physiological symptoms.

The present study was also designed to assess the effects of individual cofactor supplementation on mitochondrial ATP synthetic capacity. An increase in ATP synthesis with CoQ_{10} supplementation in control lymphocytes was demonstrated. In addition to an increase in mitochondrial ATP production with CoQ_{10}, a marked increase in non-mitochondrial ATP synthesis was observed in these investigations. It is not known whether this extra-mitochondrial role of CoQ_{10} would enable an individual to maintain an adequate energy capacity to compensate for the decline in OXPHOS function.

The incubation of control lymphocytes with vitamin B complex, carnitine, and vitamin K_1, individually or in combination, did not result in a significant increase in ATP synthetic capacity. It is reasonable to assume that many of the cofactors will only be effective if a specific enzyme defect exists. Because control lymphocytes were used in this study, the effect of

individual cofactor treatment in the lymphocytes of patients with a specific OXPHOS defect was not evaluated. Incubation of patient lymphocytes with each of the cofactors would further enhance our knowledge of possible benefits. In addition, whether dietary administration of these cofactors raises their concentration in the cell is not known. Methods to selectively target cofactors to the mitochondria by covalent binding to lipophilic carriers (Kelso et al. 2002) may enhance uptake and improve effectiveness. Further work using mitochondrially-targeted compounds may have applications in decreasing the deleterious effects of mitochondrial dysfunction associated with OXPHOS disorders.

It has been estimated that 1% to 2% of the electrons released from the respiratory chain are diverted into the formation of superoxide radicals (Robinson 1998). Oxygen free radical production by mitochondria may be one of the major triggers of apoptosis, but the significance of mitochondrial cell damage and cell death is unclear (Kelso et al. 2002). It is well recognized that the production of oxygen free radicals is increased in cells with defects in OXPHOS (Robinson 1998; Raha and Robinson 2001). The contribution of oxygen free radical damage and oxidative stress on mitochondrial function remains poorly understood. It may be that antioxidants such as CoQ_{10}, vitamin E, ascorbate, and vitamin K could slow the process of oxidative damage, although the benefit of these antioxidants over time is difficult to measure.

Provided safe dosages are used, there is the potential for benefit, and minimal harm, in attempting high-dose nutrient therapy for patients with mitochondrial disorders. The ease of administration, the nominal cost, and the low level of risk are all advantages, despite the lack of a demonstrable clinical improvement. The ability to use circulating lymphocytes to evaluate the effectiveness of therapy should also allow clinicians to design treatment strategies for individual patients.

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APPENDIX I

PARENT/ PATIENT INFORMATION

Project: **Use of NMR Spectroscopy and ATP Synthesis to Assess Cofactor Treatment in Mitochondrial Disorders**

Investigators: Barbara Marriage, MSc, RD
Thomas Clandinin, PhD
JS Bamforth, MB, MRCP(UK), FCCMG, DABMG, FRCPC

Background:

Mitochondria are small complex structures which have been called the power house of the cell because they produce most of the energy which we need to grow and live. Mitochondrial diseases occur when the mitochondria are not working properly and energy production decreases. Vitamins used as cofactors have been tried in an attempt to increase energy production. The cofactor treatment involves the supplementation of Coenzyme Q_{10}, vitamin K, ascorbic acid, B vitamins and carnitine, all of which have been reported to improve clinical symptoms. The improvement in clinical symptoms is due to an increase in energy production but improvement has been difficult to assess.

Procedures:

You or your child will be supplied cofactor treatment which will be fed in divided dosages throughout the day. You will be instructed to consume well-balanced, nutritionally adequate meals and to avoid periods of fasting. Food intake will be recorded for three days prior to clinic visits at 0, 3, 6 and 12 months. Nuclear magnetic resonance (^{31}PMR) measures energy produced by the muscle. ^{31}P-NMR spectroscopy will be performed at baseline (before starting treatment), 3, 6 and 12 months. You or your child will be laid flat with both legs inserted in the inside of the magnet. The procedure will take approximately 40 minutes. There are no known harmful effects of this technique and the examination is non-invasive and painless. A tour of the NMR facility will be arranged before the procedure is done. An additional consent form to ensure that any material that can be magnetised has been removed will be completed with the investigator before each procedure. Blood samples (approximately 5 cc) will be taken before starting the cofactor treatment and at subsequent clinic visits at 3, 6 and 12 months. This amount of blood is collected for routine clinical monitoring. An additional approximately 5 cc of blood will be collected to examine the amount of energy (ATP) that the cells produce.

Benefits:

While participating in the study, you or your child will receive the cofactor treatment free of charge. You or your child will receive the added benefit of in-depth careful evaluation. The overall benefit of the study is to assess cofactor treatment in mitochondrial disorders.

Risks:

No risks are anticipated from the study procedures.

Study Termination:

You should understand that both you and the investigator may end your or your child's participation in this study at any time, if judged appropriate, due to non-compliance with study procedures, or concerns for the best interests of you or your child. You are voluntarily allowing yourself or your child to participate in this study, and may withdraw at any time without prejudice or loss of any care or benefits to which you or your child is otherwise entitled. If any knowledge gained from this or any other study becomes available which could influence your decision to continue in this study, you will be promptly informed.

Confidentiality:

Personal records relating to this study will be kept confidential. Any report published as a result of this study will not identify you or your child by name.

Further Information:

If there are any questions regarding this study or if you feel you or your child experiences an adverse effect, contact Barbara Marriage at (780) 492-1124, Dr Thomas Clandinin at (780) 492-5188 or Dr JS Bamforth (780) 407-7333.

If you have further concerns about any aspect of this study, you may contact the Patient Concerns Office of the Capital Health Authority at (780) 407-7358. This office has no affiliation with the study investigators.

CONSENT FORM (TO BE COMPLETED BY THE PARENT OR GUARDIAN)

Title of Project:	**Use of NMR Spectroscopy to Assess Cofactor Treatment in Mitochondrial Disorders**	
Principal Investigators:		
Barbara Marriage, MSc, RD	Phone #: 492-1124	
Thomas Clandinin, PhD	Phone #: 492-5188	
JS Bamforth, MB, MRCP(UK), FCCMG, DABMG, FRCPC	Phone #: 407-7333	
	Yes	**No**
Do you understand that you have been asked to be in a research study?	☐	☐
Have you received and read a copy of the attached Information Sheet?	☐	☐
Do you understand the benefits and risks involved in taking part in this research study?	☐	☐
Do you understand that you are free to withdraw from the study at any time, without having to give a reason and without affecting your future medical care?	☐	☐
Has the issue of confidentiality been explained to you, and do you understand who will have access to your medical records?		
Do you want the investigator(s) to inform your family doctor that you are participating in this research study?	☐	☐
Who explained this study to you? _____	☐	☐

I voluntarily give consent for my child, _____,
to participate in the study described above.

Signature of Parent or Guardian: _____

(Printed Name): _____ Date: _____

Signature of Witness: _____

Signature of Investigator or Designee: _____

The information sheet must be attached to the consent form and a copy given to the parent or guardian.

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Edmonton

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Dr George Goldsand
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Research
Associate Dean
Dr Joel H Weiner
492-9723

Assistant Dean (Research and Faculty Affairs)
Dr William (Bill) A McBlain
492-9720

Admissions & Undergraduate Education
Associate Dean
Dr Anil H Walji
492-9533

HEALTH RESEARCH ETHICS ADMINISTRATION BOARD

ETHICS APPROVAL FORM

Date: October, 1997

Name(s) of Principal Investigator(s): Dr. T. Clandinin

Department: Agricultural, Food and Nutritional Science

Title: Use of NMR Spectroscopy to determine level of cofactors required to treat Mitochondrial disorders.

The Research Ethics Board has reviewed the protocol involved in this project which has been found to be acceptable within the limitations of human experimentation. The REB has also reviewed and approved the patient information material and consent form.

Specific Comments:

Signed - Chairman of Research Ethics Board

University of Alberta

This approval is valid for one year.

Issue #2433

APPENDIX II

CLINICAL HISTORY OF SUBJECTS

LHON Patients

RS This 46 year old male experienced loss of visual acuity in the right eye in 1989, at the age of 33. Deterioration continued and 6 months later visual acuity in his left eye declined. Vision loss continued until 1993 with vision noted to be 20/200. Molecular analysis confirmed that the patient had a homoplasmic mutation at nucleotide 11778 of the mitochondrial genome. In 1998 he observed weakness in his right leg and increasing fatigue with exercise. Examination revealed a slight weakness in the lower limbs with brisk knee and ankle reflexes and extensor plantar reflexes. A MRI done in 1999 showed multiple white matter changes described as being most consistent with a diffuse demyelinating process such as multiple sclerosis. Multiple sclerosis-like symptoms have been described in association with the LHON 11778 mutation. Screening of the mtDNA did not reveal any secondary mutations. A repeat MRI done in 2001 does not show any further deterioration and vision remains at 20/200. The patient reports an increase in exercise tolerance and energy level since therapy.

DB This 41 year old male had a sudden loss of vision over a period of two weeks at the age of 30. In 1986 he underwent a subtotal thyroidectomy for thyroid cancer, and is now treated with replacement therapy. Molecular analysis in 1990 confirmed a homoplasmic LHON mutation at nucleotide 11778. In 1993 he was diagnosed with obsessive

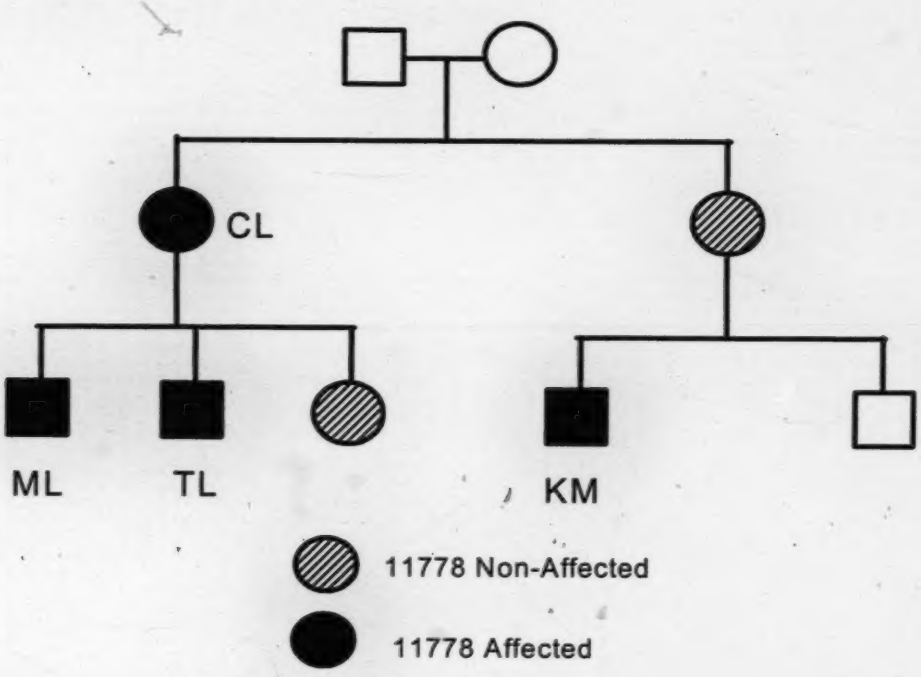
compulsive disorder and placed on psychiatric medications. For the past year he has remained on Anafril, Lithium and Clonezapam. His vision in 1993 was 20/400 in both eyes and has remained unchanged when reassessed in 2001. He does not experience any muscle or balance problems and a MRI done was reported as normal.

CL* This 46 year old woman was noted to have vision problems at age two. At age five she was diagnosed with optic atrophy. She completed school using a monocular telescope and handheld magnifiers. When examined at 26 years of age, she was noted to have vision of 20/200 in either eye. Bilateral optic atrophy was noted with no other evidence of retinopathy. Molecular analysis at age 45 confirmed that the patient was heteroplasmic for the LHON 11778 mutation. At age 46, there had been no deterioration in her vision or change in the clinical examination. An MRI done in 2002 was reported as normal.

ML* This 15 year old male (son of CL) had normal vision until age 14 (November 2000), when he experienced a sudden loss of vision over a two month period. Molecular analysis at this time revealed a heteroplasmic LHON 11778 mutation. An MRI was performed to rule out any coincident neurological disease. His visual acuity remains at 20/400 in both eyes.

TL* This 14 year old boy (son of CL) was noted to have vision problems at age three. At that time, he was diagnosed with acquired optic atrophy and vision was assessed as 20/300 in both eyes. At age 13, when family studies were done, a heteroplasmic LHON 11778 mutation was confirmed. Assessment at age 14 indicates no change in visual acuity.

KM* This nine year old boy (nephew of CL) was first noted to have vision problems at age five. Vision was assessed as 20/200 in both eyes. There was no evidence of abrupt loss of vision, similar to CL and TL. He is also heteroplasmic for the LHON 11778 mutation. Visual acuity remains unchanged since age five.



*In further investigation of family members it was found that the 10 year old daughter of CL and the mother of KM are heteroplasmic for the LHON

11778 mutation, but are not affected. This family is unique in that the prevalence of heteroplasmy in the LHON 11778 mutation is rare. In a recent study examining 167 unrelated LHON families the incidence of heteroplasmy in the 11778 mutation was found to be 5.6% (Jacobi et al. 2001).

Jacobi FK, Leo-Kottler B, Mittelviefhaus K, Zrenner E, Meyer J, Pusch CM, Wissinger B (2001) Segregation patterns and heteroplasmy prevalence in Leber's hereditary optic neuropathy. *Invest Ophthalmol Vis Sci* **42**:1208-1214

CPEO Patients

LS This 44 year old male initially presented in August 1997 with balance problems and tinnitus. A MRI done at the time reported "three small non-specific foci of white matter disease within the right cerebral hemisphere". He continued to have balance problems, progressive hearing loss, and developed muscle weakness and generalized fatigue. In November 1999 he presented to the emergency department with a hypertensive crisis (blood pressure 235/200). He began experiencing vision problems and a referral to an ophthalmologist revealed a vision of 20/25 in the right eye and 20/200 in the left eye accompanied by ophthalmoplegia and slight ptosis. A muscle biopsy in May 2000 showed a deletion of approximately five kilobases. In September 2000 the patient had an acute, complete loss of hearing with subsequent cochlear implants. Clinical examination in 2001 indicated a stabilization of his vision, ptosis and ophthalmoplegia, with proximal muscle weakness and

muscular pain. His medications include Metoprolol for hypertension and Gabapentin for muscular pain management.

PD In 1994, this 47 year old male developed rhabdomyolysis with myoglobinuria and subsequent acute renal failure after extreme exertion. He received dialysis and renal function returned to normal. In retrospect, the patient had noticed previous episodes of dark colored urine in association with intense muscular activity. A muscle biopsy revealed ragged red fibers and a seven kilobase deletion with 68% mutant mtDNA. Further investigations indicated ptosis, ophthalmoplegia and a mild sensorineural hearing loss. He has a history of hypertension and was diagnosed with hyperlipidemia in 2000. Further cardiac evaluation was performed to rule out a cardiomyopathy. He continues to have marked muscle pain associated with minimal exertion. His medications include Monopril, Metoprolol, Norvasc, Hydrochlorthiazide, Amitriptyline and Gabapentin.

LO This 36 year old female initially noticed droopy eyelids and difficulty with eye movements in her early teens. She also noticed considerable fatigue in comparison to people her age. At age 27, a muscle biopsy revealed a five kilobase deletion in the mtDNA and the presence of ragged red fibers. A MRI did not detect any cerebellar abnormalities. Nerve conduction studies did no show any evidence of peripheral neuropathy. Ophthalmological examination in 2001 indicated a stabilization of bilateral

ptosis and ophthalmoplegia. Clinical examination showed mild, diffuse muscle weakness. The patient reports an increase in energy level and exercise tolerance on cofactor therapy.

MELAS Patient

DH This 26 year old female was diagnosed with the 3243 MELAS mutation in leukocytes after investigation of a multisystem disorder in her 61 year old mother in 1998. The clinical picture in the mother included progressive hearing loss, chorioretinal dystrophy, Type II diabetes, lactic acidosis, stoke-like episodes and dementia. The daughter had experienced hearing problems since age 15, child onset migraines, poor exercise tolerance and hypertension was diagnosed at age 25. Blood work done in 1998 indicated a marginally elevated lactate without hyperalaninemia. A MRI of the head done at this time was normal. Clinical evaluation in 2001 revealed a slight deterioration in low frequency hearing with a subjective increase in energy level and exercise tolerance while on therapy.

NARP Patient

CP This 58 year old female was diagnosed with the T8993G NARP mutation in 2001 after referral to the Neurology Department, University of Alberta Hospital, with complaints of poor balance. She has retinitis pigmentosa with progressive loss of vision, ataxia, neurogenic muscle weakness with peripheral neuropathy and sensorineural hearing loss. In

retrospect, the vision problems and weakness in lower limbs started in her early twenties. She was diagnosed with hyperthyroidism and osteoporosis in 2001, and is being treated with Tapasol and Fosamax.

Cytochrome *c* Oxidase Deficiency Patient

SA This twelve year old male presented at two years of age with lactic acidosis associated with a viral illness. Enzyme analysis in muscle and skin fibroblasts revealed a deficiency of cytochrome *c* oxidase (COX). Complex I activity was slightly low in muscle but normal activity was reported in skin fibroblasts. Screening of the mitochondrial genome failed to detect any mtDNA abnormalities. A MRI done in 1993 reported "extensive bilateral and symmetrical signal changes within the basal ganglia". A repeat MRI done in 1998 was unchanged. At eight years of age he developed seizures which are treated with Gabapentin. This child has a mitochondrial encephalomyopathy with a seizure disorder, growth failure, ataxia and developmental delay. Lactic acidosis occurs with viral illness, with lactate levels within the normal range otherwise. He remains stable and shows no evidence of deterioration of his condition.

CURRICULUM VITAE
BARBARA JEAN MARRIAGE

BIOGRAPHICAL DATA

Date of Birth: August 9, 1955 Place of Birth: Woodstock, Ontario
Citizenship: Canadian Marital Status: Married; No children

Business Address: Medical Genetics Clinic
8-53 Medical Sciences Building
University of Alberta Hospitals
Edmonton AB Canada T6G 2H7
Business Telephone: (780) 492 -1124
Business Fax: (780) 407- 6845
E-mail: bmarriag@ualberta.ca
barbara.marriage@abbott.com

Home Address: 6503 - 148 Street
Edmonton AB CanadaT6H 4J4

Home Telephone: (780) 435-2443

EDUCATION

Undergraduate: University of Guelph, BSc (Applied Human Nutrition) 1978

Dietetic Internship: University of Alberta Hospital 1979

Graduate: University of Alberta, MSc (Nutrition) 1987
MSc Thesis: The Effect of Energy Intake, Body Fat Content and Food Deprivation on Hypothalamic Norepinephrine Metabolism

University of Alberta, PhD Candidate 1996 – present
PhD Thesis: Effect of Cofactor Treatment in Oxidative Phosphorylation Disorders

ACADEMIC AWARDS

Ontario General Proficiency Award 1974
Mary Ecclestone Graduate Award 1986
Province of Alberta Graduate Fellowship 1998 – 1999
Alberta Heritage Foundation for Medical Research Studentship 1998 – 2000

EMPLOYMENT AWARDS

Recognition Award – Consistent, Excellent Performance 1995
University of Alberta Hospital

EMPLOYMENT EXPERIENCE

Clinical and Administrative Dietitian 1979 – 1985

Aberhart Centre, University of Alberta Hospitals, Nutrition and Food Services
Nutritional management of patients with tuberculosis, spinal cord injuries, chronic respiratory disease, geriatrics and eating disorders. Responsible for staff involved with patient food service (20 dietary aides, 1 food clerk, 1 dietary technologist, 1 clinical dietitian)

Clinical Research Dietitian 1986 – 1994

University of Alberta Hospitals, Nutrition and Food Services
Responsible for the nutritional management of endocrinology patients and gastroenterology research studies on the Clinical Investigation Unit. Administration and nutrition consultant for the Home Enteral and Parenteral Program of Northern Alberta. Nutritional management of patients with inborn errors of metabolism.

Clinical Research Dietitian and Genetic Associate 1994 – 2002

Department of Pediatrics, Division of Medical Genetics
Nutritional management and genetic counselling for patients and families with inborn errors of metabolism.

Research Scientist, Metabolic Diseases 2002 – present

Medical Department, Abbott Laboratories
Coordination and monitoring of multi-site clinical research. Consultation and education to internal and external clients concerning nutrition management of inherited metabolic disorders.

TEACHING AND RELATED ACADEMIC ACTIVITIES

Guest Lectures

1989 – 1994	NUR 366 (Nursing)	Nutritional Assessment Altered Nutritional Requirements in Disease States
1992 – 2001	MED 413 (Medicine)	Assessment of Nutritional Status
1992 – 1994	FDNU (Foods & Nutrition)	Nutrition and Physical Activity Nutrition and Exercise Nutrition and Athletic Competition
1993 – 1995	PESS 314 (Physical Education & Sport Studies)	Nutrition and the Athlete

1994 – 1997	NUFS 452 (Nutrition & Food Science)	Inborn Errors of Metabolism
1994	HUNU 407 (Nutrition & Disease, UBC, Vancouver BC)	Inborn Errors of Metabolism
1998 – 2001	NUFS 476	Phenylketonuria
1997 – 2001	GEN 418	Phenylketonuria

Sessional Lecturer

1995	NUFS 468 (Nutrition & Food Science)	Clinical Dietetics
1999	NUFS 468 (Nutrition & Food Science)	Clinical Dietetics
2001	AFNS 500 (Nutrition)	Nutrition and Athletic Performance

Clinical Teaching of Pediatric Residents, Students and Dietetic Interns 1986 – 2002

Committee Membership for Graduate Degrees

Michelle Pluhator 1994 – 1996
(MSc – Department of Medicine)
Evaluation of Trace Element Stability in Parenteral Nutrition Solutions

Alex Sanderman 1993 – 1995
(MSc – Department of Physical Education and Sport Studies)
The Effect of Dietary Fat on Luteinizing Hormone and Cortisol Levels

INTERNAL COMMITTEE MEMBERSHIP

Nutrition Support Service Committee	1986 – 1994
Chairperson	1990 – 1994
Pediatric Medicine Program Advisory Committee – Children's Health Centre	1993 – 1995
Pharmacy and Therapeutics Committee – University of Alberta Hospitals	1987 – 1995

PROFESSIONAL MEMBERSHIP

College of Dietitians of Alberta	1988 – present
Chairperson – Sport Nutrition Interest Group	1989 – 1993
American Dietetic Association	
Nutrition Research Dietetic Practice Group	1991 – present
Sports and Cardiovascular Nutritionists	1990 – present
American Society for Enteral and Parenteral Nutrition	1995 – 1997
Canadian Dietetic Association	1979 – present
Conference Planning Committee	1987 – 1988
Fellowship Committee (Reviewer)	1992 – 1997
Garrod Association of Canada	1995 – present
Sport Medicine and Science Council of Canada	
Sport Nutrition Advisory Committee	1992 – 1998
Chairperson	1994 – 1998
Registry Sub-Committee	1994 – 1998
Resource Development Committee	1992 – 1998
Canadian Registry of Sports Nutritionists	1992 – 1998
Sport Medicine Council of Alberta	1990 – present
Board of Directors (Member at large)	1998 – 2000
Vice President	1999 – 2000
President	2000 – 2001

PROFESSIONAL SERVICE AND EXPERIENCE

Reviewer

Canadian Dietetic Association Journal
Joint Position Paper ADA/CDA – Nutrition for physical fitness and athletic performance.
Canadian Foundation for Dietetic Research – Grant Review Committee
Edmonton Diet Therapy Manual
BC Health Research Foundation – Grant Applications
SNAC-PAC – Publication of Sport Nutrition Advisory Committee (Editorial Board)

Sport Nutrition Consultant

Development and delivery of a 4 hour course on Sports Nutrition (Sport Medicine Council of Alberta)	1992 – 2002
Master Course Conductor involved in training of sport nutritionists to deliver Sport Nutrition Advisory Council (SNAC) workshops to athletes and coaches	
Sport nutrition consultant for a variety of teams and individuals at local, provincial and national level. Involved in lectures, workshops and individualized counselling	1982 – 2002
University of Alberta Fitness Unit – Varsity athletes, staff, coaches	2000 – 2002

RESEARCH GRANTS

1991 – 1993	Ross Laboratories Canadian Collaborative Pediatric Prevalence Study of Iron Deficiency Anemia (Dr A Jones, **B Marriage**)	$ 20,390
1992 – 1993	Sport Science Association of Alberta Skeletal Muscle Metabolism in Amenorrheic and Eumenorrheic Athletes (Dr V Harber, Dr S Peterson, **B Marriage**)	$10,890
1993 – 1994	University of Alberta Central Research Fund The Effect of Dietary Fat on Luteinizing Hormone and Cortisol Levels (Dr V Harber, Dr D Cumming, **B Marriage**)	$ 8,726
1993 – 1998	Ross Laboratories Growth, Tolerance, Plasma Amino Acids and Plasma Biochemistries of Infants and Toddlers with Urea Cycle Disorders and PKU (Dr P Ferreira, **B Marriage**)	$17,000
1993 – 1995	Clintec Nutrition Company Caremark Evaluation of Trace Element Stability in Parenteral Nutrition Solutions Using Inductively Coupled Plasma Mass Spectroscopy (Dr R Fedorak, **B Marriage**, M Pluhator)	$ 3,000 $15,000
1994 – 1995	Ross Laboratories Tolerance of Healthy Term Infants to Powdered Formulas Containing Whey Protein (**B Marriage**, Dr L Casey)	$ 8,000
1995 – 1997	Abbott Laboratories Effect of a New Infant Formula on Morbidity (**B Marriage**, Dr P Ferreira)	$15,525
1996 – 1999	Canadian Fitness and Lifestyle Research Institute(CFLRI) Treatment of Childhood Obesity (Dr L McCargar, **B Marriage**, Dr D Marshall, Dr S Crawford)	$30,423
1997 – 1998	Northern Alberta Children's Hospital Foundation Treatment of Childhood Obesity (Dr L McCargar, **B Marriage**, Dr D Marshall)	$13,700

BOOKS

Marriage B, Schnurr H. Sports Nutrition Resource Manual, Sport Medicine Council of Alberta, Alberta Sport Council, ISBN 0-9694952-4-2, (80 pages) 1992.

Marriage B, Schnurr H, Carter-Erdman KA, Reading K. Sports Nutrition Resource Manual, 2nd Edition, Sport Medicine Council of Alberta,ISBN 0-9694952-6-9 (184 pages) 1999.

PUBLICATIONS IN PEER REVIEWED JOURNALS

Marriage B, Johnston J. Hypothalamic norepinephrine synthesis rate in overfed, underfed and fasted mice. Pharmacology, Biochemistry and Behavior 32: 101-110, 1989.

Thomson ABR, Pinchbeck BR, Kirdeikis P, Zuk L, **Marriage B**. Night-time famotidine 40 mg is equivalent to ranitidine 300 mg in 24-hour intragastric pH in non-smoking, healthy, male volunteers. Aliment Pharmacol Ther 3(2): 199-210, 1989.

Koo W, Ke J, Tam YK, Finegan B, **Marriage B**. Pharmacokinetics of ampicillin during parenteral nutrition. J Parenteral and Enteral Nutrition 14(3): 279-282, 1990.

Cederberg C, Thomson ABR, Mahachai V, Westin J-A, Kirdeikis P, Fisher D, Zuk L, **Marriage B**. Effect of intravenous and oral omeprazole on 24-hour intragastric acidity in duodenal ulcer patients. Gastroenterology 103: 913-918, 1992.

Thomson ABR, Babiuk L, Kirdeikis P, Zuk L, **Marriage B**, Bowes K. A dose-ranging study of ranitidine and its effect on intragastric and intra-oesophageal acidity in subjects with gastroesophageal reflux disease. Aliment Pharmacol Ther 8: 443-451, 1994.

Jamali F, Thomson AB, Kirdeikis P, Tavernini M, Zuk L, **Marriage B**, Simpson I, Mahachai V. Diurnal variation in the pharmacokinetics of nizatidine in healthy volunteers and in patients with peptic ulcer disease. Journal of Clinical Pharmacology 35(11):1071-5, 1995.

Thomson A, Kirdeikis P, Jamali F, Tavernini M, Zuk L, **Marriage B**, Simpson I, Mahachai V. Effect of nizatidine and ranitidine on 24-hour intragastric pH, pepsin, prostaglandin E2 and serum gastrin concentrations in healthly volunteers. Gastroenterology & Hepatology 10(5):546-54, 1995.

Thomson AB, Mahachai V, Bailey RJ, Kirdeikis P, Zuk L, **Marriage B**, Simpson I, Jamali F. Twice daily nizatidine or ranitidine is superior to once daily dosing in elevating 24 h intragastric pH in patients with duodenal ulcer disease. J Gastroenterology & Hepatology. 11(12):1171-6, 1996.

Pluhator-Murton MM, Fedorak RN, Audette RJ, **Marriage BJ**, Yatscoff RW. Extent of trace-element contamination from simulated compounding of total parenteral nutrient solutions. American Journal of Health-System Pharmacy 53(19):2299-303, 1996.

Acosta PB, Yannicelli S, **Marriage B**, Mantia C, Gaffield B, Porterfield M, Hunt M, McMaster N, Bernstein L, Parton P, Kuehn M, Lewis V. Nutrient intake and growth of infants with phenylketonuria undergoing therapy. J Pediatr Gastroenterol Nutr 27:287-291, 1998.

Acosta PB, Yannicelli S, **Marriage B**, Steiner R, Gaffield B, Arnold G, Lewis V, Cho S, Bernstein L, Parton P, Leslie N, Korson M. Protein status of infants with phenylketonuria undergoing nutrition management. J Am Coll Nutr 18:102-107, 1999.

Pluhator-Murton MM, Fedorak RN, Audette RJ, **Marriage B**, Yatscoff RW, Gramlich LM. Trace element contamination of TPN:1 contribution of component solutions. J Parenteral and Enteral Nutrition 23:222-227, 1999.

Pluhator-Murton MM, Fedorak RN, Audette RJ, **Marriage B**, Yatscoff RW, Gramlich LM. Trace element contamination of TPN:2 effect of storage duration and temperature. J Parenteral and Enteral Nutrition 23: 228-232, 1999.

Reading KJ, McCargar L, **Marriage B**. Nutrition knowledge of adolescent and young adult hockey players. Cdn J Dietetic Prac Res 60: 166-169, 1999.

Edwards MC, Johnson JL, **Marriage B**, Graf TN, Coyne KE, Rajagopalan KV, MacDonald IM. Isolated sulfite oxidase deficiency: review of two cases in one family. Ophthalmology, 106:1957-1961, 1999.

Marriage BJ, Clandinin MT, Glerum DM. Nutritional Cofactor Treatment in Mitochondrial Disorders. J American Dietetic Association (accepted) September 2002.

Marriage BJ, Clandinin MT, MacDonald IM, Glerum DM. The Use of Lymphocytes to Screen for Oxidative Phosphorylation Disorders. Analytical Biochemistry (accepted) October 2002.

Articles Submitted

Marriage BJ, Clandinin MT, MacDonald IM, Glerum DM. Cofactor Treatment in Oxidative Phosphorylation Disorders. BBA September 2002.

PUBLICATIONS IN NEWSLETTERS, BOOKS AND NON-REFERRED JOURNALS

Marriage B. Choosing a high carbohydrate diet for athletes on the road. Sport Medicine Council of Alberta - Pulse Vol 3 No 4, 1990.

Marriage B. Protein requirements and amino acid supplementation in athletes. Sport Medicine Council of Alberta - Pulse Vol 5 No 2, 1991.

Marriage B. Foods for Success - Active people need high carbohydrate foods. Family Health, Summer 1991.

Marriage B. Sports Nutrition. O.N.E. Bulletin (Organization for Nutrition Education) Vol 12 No 2, 1993.

Marriage B. Nutritional Issues for Athletes with Disabilities. VISTA '93 - The Outlook. ISBN 0-9699102-0-7 (582 pages) 1994.

Marriage B. Physical Activity and Nutrition Programs in Alberta. The Annual Report of the Sport Science Committee of Hokkaido Vol 22, 2002.

Marriage B. Eat & Run – Carbo loading before, during and after your run. Sport Medicine Council of Alberta - Pulse Vol 15 No 1,2002.

ABSTRACTS AND PRESENTATIONS

* **Marriage B**, Johnston J. Role of norepinephrine in feeding behavior. Proceedings of the 30th Annual Meeting of the Canadian Federation of Biological Societies, 1987. (Winnipeg, Manitoba)

* **Marriage B**, Johnston J. Effects of energy intake, body fat content on hypothalamic norepinephrine synthesis. J Can Diet Assoc 48(3), 1987. (Winnipeg, Manitoba)

* **Marriage B**. Nutrition and Athletic Performance. Proceedings from a National Symposium on Wheelchair Track and Road Racing June 1988. (Edmonton, Alberta)

* Ferreira P, **Marriage B**. Arginosuccinic Aciduria. Grand Medical Rounds, Royal Alexandra Hospital, October 1988. (Edmonton, Alberta)

* **Marriage B**, Ferreira P. New approaches to treatment in glycogen storage disease. Western Canadian Investigators of Inborn Errors of Metabolism Meeting, June 1988. (Edmonton, Alberta)

* **Marriage B**, McCoy E. Hereditary fructose intolerance. Western Canadian Investigators of Inborn Errors of Metabolism Meeting, June 1988. (Edmonton, Alberta)

Koo W, Ke J, Finegan B, **Marriage B**, Tam YK. Pharmacokinetics of ampicillin during parenteral nutrition. 13th Clinical Congress of American Society for Parenteral and Enteral Nutrition, Miami FL, 1989. JPEN Vol 13(1) 1989.

Mahachai V, Thomson ABR, Westin J-A, Kirdeikis P, Fisher D, Zuk L, **Marriage B**, Cederberg C. Comparison between the effects of intravenous and oral omeprazole on 24 hour intra gastric acidity in duodenal ulcer patients in remission. J Gastroenterol Hepatology 4(3): 44, 1989.

Thomson ABR, Mahachai V, Westin J-A, Kirdeikis P, Fisher D, Zuk L, **Marriage B**, Cederberg B. Comparison between the effects of intravenous and oral omeprazole on 24 hour intragastric acidity in duodenal ulcer patients in remission. Clin Invest Med 12(4): B42, 1989.

McCoy E, **Marriage B**. Neonatal Detection and Long Term Management of Hypertryptophanemia. Am Ped Society, 1989.

Thomson ABR, Mahachai V, Westin J-A, Kirdeikis P, Fisher D, Zuk L, **Marriage B**, Cederberg C. Comparison between the effects of intravenous and oral omeprazole on 24 hour intragastric acidity. Can J Gastroenterol. 4(1): 46, 1990.

* **Marriage B**. Brion S. Development of Patient Education Material and Monitoring Tools for Home Nutritional Support Programs. J. Can Diet Assoc Vol 51(3) 1990. (Ottawa, Ontario)

* **Marriage B**. Nutritional Issues for Athletes with Disabilities. Vista 93 International Conference on High Performance Sport for Disabled Athletes May 1993 (Jasper, Alberta)

Thomson ABR, Kirdeikis P, Zuk L, **Marriage B**, Simpson I, Mahachai V. Nightime Nizatidine and Ranitidine lower pepsin activity and increase gastric juice pH and PGE 2 concentrations in healthy volunteers. Am College of Gastroenterology Meetings, Oct 1993. (New York)

Acosta PB, Yannicelli S, Arnold G, Porterfield M, Ferreira P, **Marriage B**, Plewinska M, Day-Salvatore D, Middelton L, Shapira E, Lewis V, Velazquez A. Adequate energy and nutrition support growth in children with a urea cycle disorder. VI International Congress, Inborn Errors of Metabolism W29.10: 175, 1994. (Milan, Italy)

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